2020

NOVEL POST-TRANSLATIONAL MODIFICATION AND FUNCTION OF FUS: THE RELEVANCE TO AMYOTROPHIC LATERAL SCLEROSIS

Alexandra Arenas
University of Kentucky, maar223@g.uky.edu
Author ORCID Identifier:
https://orcid.org/0000-0002-5340-5787
Digital Object Identifier: https://doi.org/10.13023/etd.2020.404

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Alexandra Arenas, Student

Dr Haining Zhu, Major Professor

Dr Isabel Mellon, Director of Graduate Studies

NOVEL POST-TRANSLATIONAL MODIFICATION AND FUNCTION OF FUS: THE RELEVANCE TO AMYOTROPHIC LATERAL SCLEROSIS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

Alexandra Arenas

Lexington, Kentucky

Director: Dr. Haining Zhu, Professor of Molecular and Cellular Biochemistry

Lexington, Kentucky

2020

[https://orcid.org/0000-0002-5340-5787]

ABSTRACT OF DISSERTATION

NOVEL POST-TRANSLATIONAL MODIFICATION AND FUNCTION OF FUS: THE RELEVANCE TO AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by the preferential death of motor neurons. Approximately 10% of ALS cases are familial and 90% are sporadic. Fused in Sarcoma (FUS) is a ubiquitously expressed RNA binding protein implicated in familial ALS and frontotemporal dementia (FTD). FUS is ubiquitously expressed in cells and has a variety of functions in the nucleus and cytoplasm. FUS mutations in the nuclear localization sequence (NLS) causes mislocalization of FUS in the cytoplasm, where it can undergo liquid-liquid phase separation and become stress granules or protein inclusions. Although FUS inclusion bodies can be found in post-mortem tissues from ALS patients, the pathological mechanism of the disease remains to be fully elucidated.

This dissertation includes two independent studies about FUS regulation and function in the cell. In the first study, we discovered a novel post-translational modification, i.e. lysine acetylation on FUS and aimed to understand whether lysine acetylation plays a role in regulating FUS function. We identified three acetylation sites in FUS: K315/K316 in the RRM domain and K510 in the NLS. We found that K510 disrupted the interaction with Transportin-1 and increased cytoplasmic protein aggregates that co-localized with stress granule markers. Interestingly, acetylation in K315/K316 reduced RNA binding to FUS and decreased the cytoplasmic inclusion formation. Similarly, treatment with deacetylase inhibitors also decreased protein aggregation in cells expressing ALS mutation P525L. Furthermore, ALS patient derived fibroblasts showed higher levels of acetylation at K510, compared to healthy controls. Finally, we demonstrated that CBP/p300 acetylates FUS, while both HDACs and Sirtuins contribute to FUS deacetylation. We concluded that FUS acetylation regulates RNA binding, subcellular localization, and inclusion formation, linking acetylation of FUS to a potential molecular mechanism for ALS/FTD.

The second study aimed to determine the function of FUS in the autophagy pathway. We found that mutant FUS did not affect autophagy flux in N2A cells. However, FUS knockout (KO) N2A cells showed a significant decrease in the basal autophagy flux compared to wild-type (WT) cells. Bafilomycin A1 treatment showed a decrease in autophagy flux in FUS KO cells, and induction of autophagy by rapamycin was not as efficient in FUS KO cells compared to WT cells. These results suggest that FUS KO affects early stages of the autophagy pathway. We found that FIP200, ATG16L1, and ATG12 gene and protein expression levels were significantly lower in FUS KO cells. FIP200 is involved in autophagy initiation and Atg16 and Atg12 form a complex to induce phagophore elongation. Overexpressing WT FUS in FUS KO cells was able to rescue gene and protein expression levels of FIP200 and ATG16L1. These findings demonstrate a novel function of FUS in regulating transcription of genes involved in early stages of the autophagy pathway. Failure to maintain protein homeostasis by protein degradation has been demonstrated in neurodegenerative diseases and finding new protein targets can be useful to discover new therapeutic options for ALS.

The two studies of this dissertation produce novel findings of FUS protein modification and function. Each study provides new insights into the role of FUS in neurodegenerative diseases including ALS and FTD, as well as novel therapeutic targets. Future studies that determine the molecular mechanisms connecting these two findings will be necessary to evaluate how FUS acetylation and inclusion formation will impact the autophagy pathway.

KEYWORDS: FUS, lysine acetylation, autophagy, stress granules, cytoplasmic inclusions, RNA-binding

Alexandra Arenas

(Name of Student)

09/08/2020

Date

NOVEL POST-TRANSLATIONAL MODIFICATION AND FUNCTION OF FUS: THE RELEVANCE TO AMYOTROPHIC LATERAL SCLEROSIS

By
Alexandra Arenas

Dr. Haining Zhu
Director of dissertation

Dr. Isabel Mellon
Director of Graduate Studies

09/08/2020
Date

DEDICATION

First, to God for giving me the strength and perseverance to continue this path even in the hardest days.

To my mother Patricia and my father Hugo, for their love, and inspiration to be the best I can in everything I do. Words are not enough to thank you for everything you have done for me.

To my sister, for her unconditional support throughout my life.

To my nieces, Juliana and Daniela, for making me the proudest 'tia' in the world.

To my beloved husband Lazaro, for his absolute support and words of encouragement throughout my career. Your persistence and energy to accomplish what you want, inspire me to be a better professional and a better person.

In loving memory of my grandma Elaine Lozano and my father-in-law Lazaro Moises de La Rosa.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my mentor and supervisor Dr. Haining Zhu for guiding me and supporting me during my career as a PhD student. His knowledge, vision, and motivation are things that will forever live with me in my professional career. I am grateful for his leadership in the development of these projects, and the freedom that he gave me helped me become an independent researcher. I also want to thank my Thesis Committee: Drs. Isabel Mellon, Edward J. Kasarskis, and Yvonne Fondufe-Mittendorf for their valuable input in our annual meetings, and their incredible support throughout these years. In addition, special thanks to Dr. Harry LeVine, III for being a mentor for the Biochemistry seminars and serving as an Outside Examiner for my final exam.

Next, I would like to thank the current members of the Zhu Lab Dr. Lisha Kuang, Dr. Jing Chen, Dr Yu Zhong, Kassandra J. Naughton and Li Liu; as well as previous members of the Lab, Dr. Marisa Kamelgarn, Dr. Sushil Dubey and Will Na. I would like to give special thanks to Dr. Jozsef Gal who took the time to train me in different experimental techniques during the first years of my PhD. To all of them, thanks for your friendship, help and support.

I would also like to thank the Department of Toxicology and Cancer Biology, including professors, students and staff, for their support during my academic career. Additionally, special thanks to the Department of Molecular and Cellular Biochemistry for allowing me to use their laboratory facilities, to

participate in their academic activities, and providing feedback about my research during student seminars. I am very grateful for the guidance both departments have provided during my academic career, and I am happy that I could have access to the knowledge from these two areas of research.

Finally, I would like to thank my entire family, my parents Hugo and Patricia, my sister Claudia, and my husband Lazaro, because I could not have done this without their support. I would also like to thank Carlos, Juliana, Daniela, Luz Dary, Rossy, Lazaro papa, Lilita, Catalina, Carroll, Janice and the rest of the family and friends in Colombia and USA who have contributed in different ways for this big accomplishment.

This work was supported by the department of Toxicology and Cancer Biology's T32 training grant, the Lyman T. Johnson Fellowship, National Institute of Neurological Disorder and Stroke grant, Department of Veteran Affairs Merit Review Award, and National Institute of Neurological Disorders and Stroke grant R21.

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Modified from the manuscript "Lysine acetylation regulates the RNA binding, subcellular localization and inclusion formation of FUS" published in Human Molecular Genetics, 21 July 2020. PMID: 32691043 https://doi.org/10.1093/hmg/ddaa159

1.1 Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by the preferential death of motor neurons. Approximately 10% of ALS cases are familial and 90% are sporadic. Fused in Sarcoma (FUS) is a ubiquitously expressed RNA binding protein implicated in familial ALS and frontotemporal dementia (FTD). The physiological function and pathological mechanism of FUS are not well understood, particularly whether post-translational modifications play a role in regulating FUS function. In this study, we discovered that FUS was acetylated at lysine-315/316 (K315/K316) and lysine-510 (K510) residues in two distinct domains. Located in the nuclear localization sequence, K510 acetylation disrupted the interaction between FUS and Transportin-1, resulting in the mis-localization of FUS in the cytoplasm and formation of stress granule-like inclusions. Located in the RNA recognition motif, K315/K316 acetylation reduced RNA binding to FUS and decreased the formation of cytoplasmic inclusions. Treatment with deacetylase inhibitors also significantly reduced the inclusion formation in cells expressing ALS mutation P525L. More interestingly, familial ALS patient fibroblasts showed higher levels of FUS K510 acetylation as compared to healthy controls. Lastly, CBP/p300 acetylated FUS, whereas both SIRT and

HDAC families of lysine deacetylases contributed to FUS deacetylation. These findings demonstrate that FUS acetylation regulates the RNA binding, subcellular localization, and inclusion formation of FUS, implicating a potential role of acetylation in the pathophysiological process leading to FUS-mediated ALS/FTD.

1.2 Introduction

1.2.1 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurological disorder characterized by the gradual degeneration of motor neurons leading to progressive weakening of muscles, paralysis and death [1]. Motor neurons are cells that reach the brain, brain stem, and spinal cord, and they communicate the nervous system with the voluntary muscles of the body [1]. In ALS, when motor neurons degenerate and die, signals from the brain are unable to reach the muscles, and as a consequence, the muscles waste away and patients lose their ability to move their arms and legs, to talk, and to breathe. Eventually, patients need respiratory support, and most people die from respiratory failure or pneumonia [2].

1.2.2 Epidemiology of ALS

According to the ALS association, more than 5,000 people are diagnosed with ALS each year, and about 16,000 people live with ALS at any

given time in the United States [3]. The National ALS registry reported that the prevalence of ALS in the United States was 5.2 per 100,000 population in 2015 [3]. The median age of ALS diagnosis is between 54 and 69 years old [4], and the median survival from the symptoms onset is approximately 2-3 years. The ratio of cases between males and females is 1.6:1 [3]. The ratio of cases between Caucasians and African Americans is approximately 2.3:1 in the United States [3]. Interestingly, there is evidence supporting that people who serve in the military have a higher risk of developing ALS [5]. In addition, smoking, head trauma, exposure to lead and other heavy metals, and exposure to agricultural chemicals are considered potential risk factors of developing ALS [6]. However, the relationship between the environmental factors and development of the disease has been difficult to identify because most of the times, the environmental exposure can change over time and may not have been accurately recorded [7].

1.2.3 Sporadic and familial ALS

ALS was initially considered a sporadic disease as Jean-Martin Charcot initially supported the notion [8]. Years later, there was a report of a family in Vermont with autosomal dominant inheritance of ALS [9]. Nowadays, it has been shown that most cases of ALS are sporadic (~ 90%), while the remaining 10% of the cases are inherited [2, 3]. The clinical characteristics of ALS are similar whether it is inherited or sporadic [10]. Studying familial cases of ALS allows to gain insights about the pathology of the disease in general, and it will

likely benefit patients with sporadic forms of ALS. Mutations in several genes have been identified to cause the familial form of ALS (fALS) [4]. For instance, an hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 gene (C9orf72) accounts for about 30%-40% of the familial cases of ALS [11, 12]. Generally, a person has 30 or fewer GGGGCC repeats. However ALS or frontotemporal degeneration (FTD) patients may carry hundreds or thousands of GGGGCC repeats, and these expansions are often associated with behavioral changes [7]. Copper-zinc superoxide dismutase (SOD1), is an antioxidant enzyme that protects cells against superoxide radicals and reactive oxygen species (ROS) [13]. About 20% of familial cases of ALS have mutations in SOD1 [2]. Many studies suggest that SOD1 mutations cause ALS in a gain-of-function manner. The first supporting evidence was done in a transgenic mouse model expressing the point mutation G93A SOD1 [14]. The mice showed motor neuron loss and became paralyzed by 5-6 months of age [14]. Additional studies of transgenic mice expressing other human SOD1 mutations show an adult-onset phenotype along with a loss of lower motor neurons [15]. Nonetheless, it was also proposed that the loss-of-function might play a minor role in the disease [16].

Another gene involved in familial ALS is *TARDBP*, the gene that encodes the TAR DNA-binding protein 43 (TDP-43). Mutations in this gene account for 4% of familial ALS and 1% of sporadic ALS cases [10]. However, approximately 97% of ALS cases, including familial and sporadic, show TDP-43-positive inclusions. The TDP-43 proteinopathy [17] is characterized by the

exclusion of TDP-43 from the nucleus and the deposition of full-length and fragmented TDP-43 in cytoplasmic aggregates that are also ubiquitinated and hyperphosphorylated [18]. TDP-43 is a DNA/RNA binding protein that has the ability to shuttle between the nucleus and the cytoplasm [19]. In the nucleus it has been reported to be involved in transcription and splicing [17, 20]. In the cytoplasm it has a role in RNA transport [21] and stress granule response [22].

It is unclear whether TDP-43 causes ALS by a gain or loss of function. A few studies show that TDP-43 overexpression mimics the disease phenotype in transgenic mice models [23, 24]. However, conditional TDP-43 KO mouse models have demonstrated that loss of TDP-43 also causes motor neuron degeneration, very similar to the human phenotype along with TDP-43 proteinopathy [25, 26]. Currently, it is considered in the field that both mechanisms may contribute to the development of the disease [17, 27].

Furthermore, mutations in Fused in Sarcoma (FUS, also called Translocated in Liposarcoma or TLS) are found in about 5% of the familial cases of ALS [28]. FUS pathology and mutations have also been reported in sporadic ALS cases [28-31]. Mutations in the FUS gene are associated with juvenile and young adult cases of sporadic ALS [32-34]. Moreover, FUS pathology is reported in approximately 10% cases of another clinically overlapping disease frontotemporal dementia (FTD-FUS) [30]. FUS is also a DNA/RNA binding protein with diverse functions in the cell [35]. Our lab focuses on the function of FUS and how mutations and post-translational

modifications affect the function of this protein and its association with the development of ALS and FTD.

1.2.4 Fused in Sarcoma (FUS)

FUS is a ubiquitously expressed RNA-binding protein that plays a role in different cellular processes such as DNA repair [36-38], transcription [39-49], RNA splicing [48, 50, 51], nucleo-cytoplasmic RNA shuttling [52] and dendritic RNA transport [53-55]. FUS contains a very transcriptionally active N-terminal prion-like domain, (QGSY-rich), an RNA recognition motif (RRM), a zinc finger domain flanked by two Arginine-Glycine-Glycine (RGG)-rich domains, a nuclear export sequence (NES), and a C-terminal nuclear localization sequence (NLS) [27] (Figure 1.1). FUS is mainly localized in the nucleus, although it is also present in the cytoplasm of neuronal cells at lower levels [28]. Many of the fALS–related FUS mutations are localized in the C-terminal NLS, causing mis-localization of FUS to the cytoplasm where it forms stress granule-like structures [29-32]. A loss of FUS function in the nucleus and a gain of toxic function in the cytoplasm can both contribute to the disease mechanism concomitantly [33].

1.2.5 Physiological roles of FUS in the nucleus

Several groups have characterized the functions of FUS in the cell. Since FUS localizes mainly in the nucleus, it has been reported to play crucial

roles in transcription, miRNA processing, splicing, and DNA damage response. For instance, FUS has been reported to bind DNA and RNA through their RRM domain, the RGG-rich domain, and the zinc finger domains [36, 56, 57]. A common ‘GGUG’ RNA consensus sequence has been identified to bind FUS [56], but more recent studies have reported that FUS can bind G/C or A/C rich regions [58, 59]. Another study confirmed that FUS binds long regions in the 3’ UTR of Nd1-L mRNA [53], suggesting that FUS can recognize other RNA binding domains from other proteins and bind to secondary structures.

Additionally, FUS also plays a role in transcription. Numerous studies have found that FUS interacts with transcription factors [49, 60] hormone receptors [61], and the transcriptional machinery that includes RNA polymerase II and the TFIID complex [62, 63]. Similarly, FUS has been shown to repress transcription of Cyclin D1 in response to DNA damage, by being recruited into the promoter region, and inhibiting the activity of the acetyltransferases CREB-binding protein (CBP) and p300 on Cyclin D1 [39]. Another study reported that FUS N-terminal QGSY-rich region is required for active-chromatin binding, and for transcription activation of MnSOD and histone-lysine *N*-methyltransferase, SMYD3. The same study added that FUS’ self-assembly is necessary for chromatin binding, and that FUS ALS mutants disrupt these functions [48]. These data show that FUS is capable of binding RNA, transcriptional machinery, and active chromatin, having a relevant role in gene transcription.

Similarly, FUS has been found to be involved in microRNA (miRNA) processing. There is evidence that miRNAs might play a protective role in motor neurons and promote motor neuron survival [64, 65]. Specifically, mice that lacked miR-218 died at birth with motor neuron defects [66]. Additionally, motor neurons derived from a spinal muscular atrophy patient showed downregulation of miR-375, and these motor neurons were highly susceptible to DNA damage [65]. Moreover, in a transcriptome analysis, miR-375 was deregulated in mutant FUS motor neurons, while toxic expression of apoptotic factor p53 and neural RNA-binding protein ELAVL4, increased significantly in FUS mutants [67]. FUS has been directly associated to miRNA biogenesis by enabling the recruitment of the enzyme Drosha to chromatin transcription sites, and by interacting with nascent pri-miRNAs, in order to allow effective miRNA processing [68]. Together, these studies show that FUS plays an important role in miRNA biogenesis, having an impact in post-transcriptional gene regulation.

Furthermore, FUS is involved in alternative splicing. Proteomics studies have found that FUS is present in the spliceosome [69, 70]. Also, sequencing analysis have showed that FUS is enriched in exons that are actively undergoing alternative splicing [59, 71]. Interestingly, a group found that FUS binds to a conserved region in exon 7 of its own pre-mRNA. [72]. The study showed that FUS overexpression caused inhibition of exon 7 splicing and downregulation of endogenous FUS protein levels, showing a mechanism for FUS autoregulation. In contrast, cytoplasmic FUS mutants were unable to

repress exon 7 splicing and regulate protein expression [72], showing that FUS nuclear localization is necessary for alternative splicing and FUS autoregulation.

Additionally, FUS interacts with alternative splicing regulators such as SMN (survival motor neuron), and U1 snRNP [73]. SMN is highly expressed in spinal cord and brainstem, and mutations in this protein cause Spinal Muscular Atrophy (SMA)[74] . SMN is part of a complex that plays an important role in mRNA processing [75]. In the cells, this protein localizes to nuclear gems, which are nuclear structures where small nuclear ribonucleoprotein (snRNP) maturation takes place [74]. The relationship between FUS and SMN has been widely studied, since these two proteins have a crucial role in neurodegeneration. Specifically, ALS-associated FUS mutations sequester SMN into cytoplasmic inclusions, inhibiting the function of SMN in the nucleus and reducing the number of nuclear gems. Similarly, HeLa FUS knockout cells also show loss of nuclear gems [73]. In a similar study, FUS ALS mutations increased the interaction between FUS and SMN, causing a decrease in number of nuclear gems and a lower levels of snRNAs [76]. These studies demonstrate that FUS plays an important role in mRNA biogenesis and splicing, and show that a gain of toxic function, along with a loss of nuclear function can occur alongside and contribute to the disease phenotype.

1.2.6 Physiological roles of FUS in the cytoplasm.

FUS has the capacity of shuttling between the nucleus and the cytoplasm, where it can regulate different facets of RNA metabolism such as protein translation, RNA stability, RNA transport, or stress granule formation. The involvement of FUS in RNA transport has been reported in various studies. For example, a proteomics study found that FUS interacts with kinesin KIF5, a protein that is part of an RNA-transporting granule [77]. Additionally, another study showed that following NMDA activation, FUS requires actin filaments and microtubules to translocate to dendritic spines and is involved in preserving spine morphology and stabilizing synaptic structure by transporting mRNAs that encode for Nd1-L, an actin-stabilizing protein. [53, 54]. These studies show that FUS has an important role in transporting RNA to active translation spots in the cytoplasm, and this function is crucial for the good development of neuronal cells.

An early study found that FUS associates with adenomatous polyposis coli (APC), and it is required for translation of APC-ribonucleoprotein (APC-RNPs) transcripts in cell protrusions [78]. They show that aggregates formed as a result of mutant FUS overexpression recruit APC-RNPs, and these aggregates are transcriptionally active. Later on, a breakthrough mass spectrometry study showed that FUS is involved in protein translation and non-sense mediated decay (NMD) [79]. They found that cytoplasmic mutant FUS inclusions contained proteins involved in translation and RNA surveillance. Furthermore, using a variety of assays, they proved that FUS ALS mutations

impaired global protein translation. Additionally, they demonstrated that translation initiation was not affected by FUS mutations. Interestingly, NMD factors were disrupted in N2A cells overexpressing FUS mutants and in ALS-patients derived fibroblasts, leading to over activation of the NMD pathway. This study suggests that FUS has an important role in protein biosynthesis and mRNA quality control.

A more recent work using proteomics compared the translated proteomes of WT and mutant FUS after being exposed to stress. They identified a great number of neurodegeneration-related proteins that become unregulated by avoiding stress induced translational repression [80]. One of the target proteins they found to be downregulated in mutant FUS cells under stress was COPB1, a protein involved in the retrograde transport between the Golgi apparatus and the endoplasmic reticulum (ER), showing that vesicular transport can also be impacted in FUS-ALS.

Cytoplasmic FUS is also involved in stress granule dynamics and inclusion formation. WT cytoplasmic FUS can be chaperoned back to the nucleus by Transportin-1 (TNPO1), through a direct interaction with FUS-NLS [81]. Meanwhile, many FUS fALS-related mutations are localized in the C-terminal NLS, decreasing the binding affinity between FUS and TNPO1, causing mis-localization of FUS to the cytoplasm where it can form inclusions that co-localize with stress granule proteins [82-85]. Moreover, a study found that patterns of aggregation in nuclear and cytoplasmic FUS are different [86]. The study showed that while nuclear FUS requires the QGSY-rich region to

form nuclear granules [87], cytoplasmic FUS did not required the prion-like domain to form mutant FUS cytoplasmic inclusions, suggesting that the structure of these aggregates are different depending on their sub-cellular localization. In fact, they showed that the nuclear compartment where FUS aggregates are formed, contribute to their distribution pattern and behavior, irrespective of WT or mutant FUS. In the same study, the authors found that the RNA binding capability of FUS is required to form cytoplasmic inclusions, and that chromatin related RNAs are crucial for FUS' oligomerization [86]. Similarly, using a Drosophila model of ALS, a group generated mutants deficient in RNA-binding. They found that RNA-binding deficient mutant FUS was not able to incorporate into stress granules [88]. Stress granules are believed to be a preceding event in inclusion formation, however, further studies are necessary to understand how these structures become aberrant for the cells, when mutant FUS is present.

1.2.7 FUS and Post-translational modifications

Post-translational modifications (PTMs) refer to covalent attachments or proteolytic cleavages of a functional group to a protein after biosynthesis, changing or regulating its subcellular functions and increasing the diversity of the proteome [89]. Common eukaryotic PTMs include methylation, phosphorylation, acetylation, ubiquitination, and sumoylation (34). Lysine acetylation occurs when the acetyl group in acetyl-CoA is transferred to the ε-amino group of a lysine residue of a protein [90]. This process is reversible,

and it is regulated by two types of enzymes: lysine acetyltransferases (HATs), that add the acetyl group to the lysine residue, and lysine deacetylases (HDACs) that remove it [90].

Lysine acetylation has many roles in the cells, however the effect of this PTM on gene expression has been well characterized. It has been demonstrated that when histone tails are acetylated, the interaction between the histones and DNA backbone is disrupted, resulting in a relaxation of the condensed chromatin, making it more accessible to the transcription machinery [91]. However, lysine acetylation can regulate many other functions in the cells. A proteomics study identified around 1750 proteins that can be acetylated [92]. They also showed that lysine acetylation can be involved in various cellular processes including RNA transcription, splicing, DNA damage and repair, nuclear transport, and others [92].

Lysine acetylation has been reported in various neurodegenerative disorders, including ALS [93-96]. For example, misfolded tau protein acetylation serves as a disease marker for Alzheimer's pathology [96]. Similarly, TDP-43 acetylation impairs RNA-binding and promotes cytoplasmic aggregation that resembles the TDP-43 pathology in ALS patients [94]. Furthermore, in a yeast study, human FUS was overexpressed, resulting in decreased acetylation of histone H3, specifically on lysine 14 (H3K14) and lysine 56 (H3K56) [97]. Additionally, these cells showed overall low RNA levels suggesting that FUS proteinopathy might be related to decreased transcription.

In addition, FUS has also been found to interact with histone deacetylase 1 (HDAC1) in neurons [98]. This interaction is important for the recruitment of HDAC1 to DNA double strand break sites in response to DNA damage. Another study found that FUS ΔNLS/ΔNLS knock-in mice showed HDAC1 mislocalization to the cytoplasm, suggesting that FUS cellular localization affects the function of its interacting partners [98].

1.2.8 Conclusion and study rationale

In summary, FUS is ubiquitously expressed in the cells and different studies have described its functions in the nucleus and the cytoplasm. On the other hand, we also know that lysine acetylation is one of the major PTMs that can regulate several functions in the cells. There is evidence by several studies that FUS can be modified by arginine methylation regulating the nuclear import of FUS, and FUS phase separation [99-101]. However, acetylation of FUS is yet to be reported and represents an understudied mechanism in ALS and FTLD-FUS pathology. In this study we asked whether FUS is modified by acetylation, what sites can be acetylated in FUS, and what are the functional consequences of FUS acetylation. The goal of this study is to find new regulators of the function of FUS that provide more information on the molecular disease mechanism, and to characterize novel targets for therapeutic approaches in ALS and other neurodegenerative disorders.

1.3 Materials and Methods

1.3.1 Cell culture and transfection

HEK293Tand N2A cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, D5796) with 10% fetal bovine serum, penicillin-streptomycin, and amphotericin B at 37°C in 5% CO2/95% air with humidification. The pCMV10-3×FLAG-FUS WT and pEGFP-C3 FUS plasmids were generated as reported [83] FUS mutations were introduced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). HEK293T and N2A cells were transfected with Polyethylenimine "Max" (Polysciences, Inc. 24765) and Lipofectamine 2000 (Thermo Fisher Scientific, 11668), respectively.

1.3.2 Immunoprecipitation (IP)

Cells were lysed with 1× RIPA buffer (Millipore Sigma, 20-188) supplemented with protease inhibitor cocktail (Millipore Sigma, P8340, 1:500), sodium orthovanadate (1 mM), and deacetylase inhibitor (DACi) cocktail (nicotinamide (20 mM), sodium butyrate (20 mM) and Trichostatin-A (1.5 μM)). Lysates were homogenized by sonication and centrifuged at 1,000g for 15 minutes at 4°C. Immunoprecipitations were performed overnight at 4°C using EZview™ Red ANTI-FLAG® M2 Affinity Gel (Millipore Sigma, F2426) or 4μg/ml mouse monoclonal ANTI-FLAG® M2 antibody (Millipore Sigma, F3165) and Protein G UltraLink Resin (Thermo Fisher Scientific, 53126). Beads were washed three times with lysis buffer and bound proteins were eluted with 0.15 μg/μL 3×FLAG peptide (Millipore Sigma, F4799) in 1× RIPA

buffer at 4°C for 1 hour. Samples were resolved by SDS-PAGE followed by immunoblotting. Where indicated, a cocktail of RNase A and RNase T1 (Life Technologies, AM2286) was added to the lysates at 1:100 dilution before the overnight immunoprecipitation.

1.3.3 Immunoblotting

Proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes (Pall, 66485). The membranes were blocked with 5% non-fat dry milk in TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween-20) or bovine serum albumin (BSA) for 1 hour. The antibodies used include: mouse anti-FUS (Santa Cruz, sc-47711), mouse monoclonal ANTI-FLAG® M2-Peroxidase (HRP) (Millipore Sigma, A8592), rabbit anti-acetylated-lysine (Cell Signaling Technology, 9441), rabbit anti-PABP1 (Abcam, ab21060), mouse anti- β -actin (Santa Cruz Biotechnology, sc-81178), rabbit anti-β-actin (Cell Signaling 8457), and a custom-made rabbit anti-K510-acetylated FUS antibody (see below).

1.3.4 Identification of FUS acetylation by Mass Spectrometry

FLAG-tagged FUS was transfected into HEK293T cells. Transfected cells were treated overnight with a cocktail of lysine deacetylase inhibitors (30 mM nicotinamide, 50 mM sodium butyrate and 3 μM Trichostatin-A) one day post-transfection in regular medium. Anti-FLAG immunoprecipitation was performed immediately after deacetylase inhibitor treatment. Eluates were run on SDS-PAGE and the gel was stained with SYPRO Ruby protein gel stain

(Thermo Fisher Scientific, S12000). The bands that corresponded to FLAG-FUS (electrophoretic mobility of approx. 72 kDa) were excised and subjected to dithiothreitol reduction, iodoacetamide alkylation, and in-gel chymotrypsin digestion. Peptides were extracted, concentrated and subjected to LC-MS/MS analysis at the University of Kentucky Proteomics Core Facility as previously reported (48). Briefly, LC-MS/MS analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent , Dublin, CA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed phase cHiPLC column (75 μm x 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid while B was acetonitrile with 0.1% (v/v) formic acid. The data-dependent acquisition method consisted of an Orbitrap MS scan (300-1800 m/z) with 60,000 resolution for parent ions followed by MS/MS for fragmentation of the 7 most intense multiple charged ions. The LC-MS/MS data were submitted to a local MASCOT server for MS/MS protein identification via Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Waltham, MA) against a custom database containing only RNA-binding protein FUS (FUS_HUMAN) downloaded from Uniprot. Typical parameters used in the MASCOT MS/MS ion searchinclude: chymotrypsin digestion with a maximum of three missed cleavages; 10 ppm precursor ion and 1.2 Da fragment ion mass tolerances; lysine acetylation; cysteine carbamidomethylation; methionine oxidation.

1.3.5 Generation of FUS KO (ΔFUS) N2A cells.

The FUS knockout cells were generated by employing CRISPR technology. N2A cells were transfected with FUS double nickase CRISPR plasmid (Santa Cruz Biotechnology, sc-433326-NIC) following the manufacturer's instructions. Clonal cell lines were isolated with serial dilution, and the FUS status of the clones was determined with immunoblotting.

1.3.6 Generation of the anti-acetylated-K510 FUS antibody.

All peptides below were synthesized with an added extra N-terminal cysteine residue to facilitate conjugation to Sulfolink resin or KLH (Genscript, Piscataway, NJ). The rabbit anti-acetylated-K510 FUS antibody was raised against the peptide antigen N-G504GFGPG(K510Ac)MDSRG515-C. The peptide was synthesized with C-terminal amidation and conjugated to Keyhole Limpet Hemocyanin (KLH) for immunization (Pocono Rabbit Farm and Laboratory, Canadensis, PA). The serum was first depleted with the non-acetylated FUS peptide N-G504GFGPGK510MDSRGEHRQDRRERPY526-C, then affinity-purified with the acetylated FUS peptide N-G504GFGPG(K510Ac)MDSRGEHRQDRRERPY526-C conjugated to Sulfolink resin (Thermo Fisher Scientific, 44999). The antibodies were eluted with 0.1 M glycine-HCl buffer, pH 2.5, neutralized by the addition of Tris-HCl, 1 M, pH 8.5, and dialyzed against storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.04% [w/v] sodium azide).

1.3.7 GST-TNPO1 purification

The human TNPO1 coding region was PCR amplified with primers 5'-CGTCGGATCCATGGAGTATGAGTGGAAACCT-3' and 5'-CGTCGTCGACTTAAACACCATAAAAAGCTGCAAGA-3' from MGC clone 4178989 as template and inserted between the BamHI and SalI sites of pGEX-6P-3 (GE Healthcare 28-9546-51). The GST-TNPO1 fusion protein was expressed in Rosetta 2 (DE3) pLysS E. coli cells (Millipore 71401) at 28°C incubation temperature, purified using Glutathione Sepharose 4B resin (GE Life Sciences, 17-0756-01) following the manufacturer's recommendations, and eluted with 15 mM reduced L-glutathione (Gold Biotechnology, G-155-25) in 100 mM Tris-HCl, pH 8.0, and 2 mM DTT. Aliquots were flash-frozen in liquid nitrogen and stored at −80°C until use.

1.3.8 GST-TNPO1 pulldown with Sulfolink-immobilized FUS peptides

The acetylated and non-acetylated FUS peptides synthesized with an added extra N-terminal cysteine residue were immobilized to Sulfolink resin following the manufacturer's instructions (Thermo Fisher Scientific, 44999). The beads were blocked with 0.5mg/mL BSA in TNPO1 buffer (20mM Na-PO4, pH7.4, 150mM KCl, 0.5mM EDTA, 5mM MgCl2, 10% glycerol, 1mM DTT, 20mM Nicotinamide) for 1h at 4°C. The indicated amounts of GST-TNPO1 were added to the blocked beads with the immobilized FUS peptides and incubated at 4°C for 3 hours with mild rotation. The beads were washed with TNPO1 buffer 3 times and eluted by the addition of 2× Laemmli sample

buffer with beta-mercaptoethanol. After incubation at 94°C for 5 minutes, the eluted samples were subjected to SDS-PAGE and Western blot.

1.3.9 Immunoprecipitation followed by qPCR

Immunoprecipitation was carried out as described above, with the difference that SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, AM2696, 0.2 U/µl) was added to the lysis and elution buffers, and incubation with the antibody was performed for 2 hours. In the last step of the immunoprecipitation, the eluted sample was divided into two aliquots. One aliquot was used for immunoblotting and the other was used for RNA isolation using TRIzol Reagent (Thermo Fisher Scientific, 15596), following the manufacturer's instructions. Equal aliquots of the isolated RNA were reverse transcribed using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, 18080). The resulting cDNA was subjected to quantitative real-time PCR using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, 4367659). The qPCR primers and annealing temperatures were: mouse FUS pre-mRNA flanking exon 7, 5'-CAACCCTTTTGTAGCCGTTGG-3' and 5'-CAGCAGGAGGCATTCTACCC-3', 59°C; and mouse RPL13A, 5'-CTGTGAAGGCATCAACATTTCTG-3' and 5'-GACCACCATCCGCTTTTTCTT-3' [102], 55°C. The qPCR results were analyzed using the ΔΔCT method.

1.3.10 Immunofluorescence and fluorescence microscopy

N2A cells were seeded on gelatin-treated coverglass and transfected with EGFP-FUS constructs. Cells were treated with the lysine deacetylase inhibitor cocktail for six hours when indicated. 48h after transfection, cells were fixed with 4% formaldehyde in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and permeabilized with 1× PBS supplemented with 0.25% [v/v] Triton-X100. The coverslips were blocked with 10% [w/v] BSA in 1× PBS for 1h, followed by incubation with primary antibody at room temperature overnight. Coverslips were rinsed with 1× PBS and incubated with secondary antibodies for 1 hour at 37°C. The primary antibody was rabbit anti-G3BP1 (Proteintech, 13057-2-AP) and the secondary antibody was Alexa Fluor 568 donkey anti-rabbit (Thermo Fisher Scientific, A10042). All the samples were stained with 4′,6-Diamidino-2-phenylindole (DAPI) and mounted with Vectashield Mounting Medium (Vector Laboratories, H-1000-10). Confocal images were acquired using a Nikon A1 confocal microscope with a 40x objective. Z-stack images were obtained from random view fields using identical parameters for all the samples. Maximum intensity projections of the Z-stacks were analyzed using ImageJ (http://imagej.nih.gov/ij) as reported previously [103]. Inclusion-positive cells were defined as any cell with one or more inclusions larger than 5 pixels. The number of inclusion-positive cells was normalized with the total number of GFP-positive cells in each view field.

1.3.11 Patient skin fibroblast isolation and culture

Human skin fibroblasts were prepared and maintained as described (78). ALS patients and healthy family members consented to donate the samples. Protocol was reviewed and approved by the Institutional Review Board of the University of Kentucky. A skin biopsy of 3 mm diameter was obtained from the subjects and the tissue was washed in phosphate-buffered saline (PBS), minced, and incubated in Minimum Essential Medum (Sigma-Aldrich, M5650) supplemented with 20% FBS, 2 mM L-glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin) at 37°C under 5%CO2/95% air. Information on the subjects examined in this study is shown in Table 1.1

1.3.12 Statistical analysis

The quantification of Western blot bands was performed using Image Lab software by BioRad. Statistical analysis was performed with SigmaPlot 14.0 software. Band intensities were calculated and comparison between groups was performed using Anova with post hoc Tuckey HSD test. Student's *t* test was used to determine statistical significance between two groups. Chi square was used to compare difference between proportions. Experiments were not blinded. Fluorescence microscopy was quantified from more than 10 different view fields and all experiments were done in triplicates.

Table 1.1. List of patient fibroblast

ID	Group	FUS genotype	Gender	Age of sample collection
017	Control	WT/WT	Female	43
010	Control	WT/WT	Female	36
012	Control	WT/WT	Female	63
008	Control	WT/WT	Female	24
089	Control	WT/WT	Male	20
091	ALS patient	WT/R521G	Female	31
014	ALS patient	WT/R521G	Male	42
007	ALS patient	WT/R521G	Male	58
018	ALS patient	WT/R521G	Female	40
090	ALS patient	WT/P525R	Female	26

1.4 Results

1.4.1 FUS is acetylated at specific lysine residues in two distinct functional domains.

To evaluate whether FUS was acetylated, we immunoprecipitated endogenous FUS from N2A cells treated with a deacetylase inhibitor (DACi) cocktail (30 mM nicotinamide, 50 mM sodium butyrate and 3 μM Trichostatin-A), followed by immunobloting with a pan-acetylated lysine antibody. A prominent acetylated FUS band was observed in the presence of DACi cocktail as compared to a significantly weaker band in the absence of DACi (Figure 1.2A). The result suggests that FUS is acetylated and the acetylation is dynamic.

Next, we used LC-MS/MS to identify the lysine acetylation sites. 3×FLAG-tagged FUS was transfected into HEK293T cells and treated with the DACi cocktail overnight. Subsequently, a FLAG immunoprecipitation (FLAG-IP) was performed, and the eluates were run on SDS-PAGE followed by SYPRO Ruby staining. FUS bands at approximately 72 kDa were excised, subjected to in-gel chymotrypsin digestion, and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described [104]. Figure 1.2B shows the mass spectrometric evidence of FUS acetylation at K510 and Figure 1.2C shows the di-acetylation at K315 and K316.

We next validated these three acetylation sites using a mutagenesis approach, in which acetylation mimicking mutants (Lys to Gln or K/Q) were generated for each site. Mutating lysine to glutamine is a method widely used in the field to study protein acetylation [94, 104] In our data, replacing lysine with glutamine at K315 or K316 alone did not result in significant loss of FUS acetylation (Figure 1.2D-E). However, when these two sites were mutated together, FUS acetylation was decreased significantly (Figure 1.2D-G). This suggests that acetylation at both sites may occur interchangeably, which is consistent with the detection of di-acetylation of K315 and K316 in the LC-MS/MS analysis. Similarly, K510Q mutant decreased FUS acetylation significantly, and mutating the three sites also showed acetylation decrease (Figure 1.2F-G). FUS lysine acetylation was not completely abrogated in the triple K315/316/510Q mutant, suggesting that additional acetylation sites might be present beyond the detection limit in our study.

Lysine residues 315 and 316 are localized in the RNA recognition motif (RRM), specifically in the very positively charged KK-loop that facilitates the electrostatic interaction between FUS and DNA/RNA (Figure 1.2H). NMR structure of the RRM domain found that K315 and K316 are inserted into the major groove of the stem-loop RNA and in contact with the ribose-phosphate backbone of both strands [105]. Previous studies have shown that replacing lysine residues by alanine in the KK-loop disrupted nucleic acid binding [57]. Thus, we hypothesized that acetylation of K315/K316 could regulate the RNA binding of FUS by a similar mechanism (Figure 1.2I). In contrast, lysine 510 is

localized in the C-terminal NLS (Figure 1.2J). The NLS of FUS interacts with the nuclear transport receptor Transportin-1 (TNPO1) at multiple positively charged residues including K510, and this interaction is crucial for nuclear localization and function of FUS [81, 85]. Our hypothesis was that acetylation at K510 disrupts the interaction between FUS and D693 of TNPO1 (Figure 1.2J) by removing the positive charge of lysine and imposing steric hindrance. The net effect was predicted to be that K510 acetylation affects the interaction with TNPO1 and thereby regulates the subcellular localization of FUS.

1.4.2 Acetylation of FUS at K315/K316 regulates its RNA binding activity.

To test the hypothesis that acetylation of K315/K316 regulates RNA binding to FUS, we first tested FUS binding to its own pre-mRNA surrounding exon 7 as previously reported [72]. The 3×FLAG-tagged FUS (WT and K-Q acetylation mimicking mutants) was immunoprecipitated and the quantity of FUS pre-mRNA was measured by reverse transcription and quantitative PCR. The K315/316Q acetylation mimicking mutant co-precipitated significantly less FUS mRNA as compared to WT FUS (Figure 1.3A). In contrast, K510Q did not change the RNA binding. As a control, we verified that the total FUS pre-mRNA levels were comparable in all cell extracts (Figure 1.3A).

A second assay was used to test FUS RNA binding by examining the interaction between FUS and poly-A binding protein (PABP1), which was reported to be RNA-dependent [83]. We performed a FLAG-IP from N2A cells

transfected with 3×FLAG-tagged WT or K/Q mutant FUS to examine the FUS-PABP1 interaction. As expected, in the presence of RNase cocktail containing a mixture of RNAse A and RNASe T1, the WT FUS-PABP1 interaction was completely abrogated (Figure 1.3B-C). When the cells were treated with DACi cocktail, the WT FUS-PABP1 interaction decreased significantly, suggesting that acetylation affected RNA binding. We also found that the PABP1-FUS interaction was affected even by single K/Q mutations in the KK-loop, and when both sites were mutated to glutamine, the interaction was impaired at similar levels as the DACi treatment itself (Figure 1.3B-C). In contrast, K510Q mutant did not affect the FUS-PABP1 interaction. Overall, these results support the hypothesis that lysine acetylation of K315/K316 impairs FUS binding to RNA.

1.4.3 The effect of FUS K510 acetylation on the FUS NLS-TNPO1 interaction.

To test the hypothesis that acetylation of K510 affects the interaction between FUS and TNPO1 and influences the subcellular localization of FUS, we employed chemically synthesized FUS-NLS containing the acetylated K510 in an *in vitro* interaction study with TNPO1. The acetylated and non-acetylated FUS-NLS peptides were immobilized to Sulfolink resin and incubated with different amounts of purified GST-TNPO1. The pulldown of TNPO1 by the non-acetylated FUS peptide was detectable with the lowest amount of 12.5 pmol TNPO1 (Figure 1.3D). However, the K510-acetylated

FUS-NLS peptide pulled down significantly less TNPO1 noting that the interaction was almost undetectable with 25 pmol of TNPO1 (Figure 1.3D-E). As a control, Sulfolink beads without FUS peptide were subjected to the same immobilization protocol and incubated with 0 or 200 pmol of TNPO1. No signal was detected at 0 pmol of TNPO1 but a weak band was detected when 200 pmol of TNPO1 was incubated with the blank beads. Thus, acetylation of K510 affected the interaction between FUS and TNPO1, suggesting that this post-translational modification in the NLS reduces the nuclear import of FUS and hence may alter cytoplasmic localization.

1.4.4 Acetylation of FUS at distinct sites differentially alter cellular localization and stress granule formation.

Since the acetylation at K315/K316 and K510 residues affects the FUS-RNA and the FUS-TNPO1 interaction, respectively, we used acetylation mimic K/Q mutants to determine whether acetylation of these lysine residues differentially affect its subcellular localization and inclusion formation by fluorescence microscopy. In Figure 1.4A, EGFP-tagged WT FUS was localized to the nuclei of N2A cells. Similarly, the K315Q/K316Q mutant was localized to the nuclei of the cells, suggesting that acetylation of K315/K316 residues did not affect FUS nuclear localization. In contrast, ~30% of the K510Q mutant showed cytoplasmic localization while ~40% of the triple K/Q mutant FUS was in the cytoplasm (Figure 1.4A-B). More interestingly, ~59%

of cells expressing the K510Q mutant FUS showed cytoplasmic inclusions whereas only ~22% of cells expressing the triple mutant K315Q/K316Q/K510Q showed cytoplasmic inclusions (Figure 1.4C, $p<0.001$). The reduced formation of cytoplasmic inclusions by the triple K/Q mutant suggests that that RNA binding is important for the formation of FUS cytoplasmic inclusions, which is consistent with our previous report [106]. Next, we assessed whether the cytoplasmic inclusions formed by acetylation mimicking mutants were colocalized with the stress granule marker G3BP1 in N2A cells using fluorescence microscopy. As expected, EGFP-FUS WT and K315Q/316Q neither formed inclusions nor co-localized with G3BP1. In contrast, EGFP-FUS K510Q and K315Q/K316Q/K510Q cytoplasmic inclusions were co-localized with G3BP1 (Figure 1.5). These results are consistent with previous studies that ALS mutant FUS forms cytoplasmic inclusions co-localized with stress granule markers [83, 86]. Taken together, we conclude that the acetylation of K510 affects the interaction between FUS and TNPO1, causing the mis-localization of FUS to the cytoplasm and the formation of stress granule-like inclusions. In addition, the acetylation of K315/K316 reduces the RNA binding and suppresses FUS-positive stress granules.

1.4.5 FUS acetylation in the RRM domain decreases the formation of ALS mutant inclusions.

To further test whether acetylation of K315/K316 in the KK-loop of the RRM domain affects the formation of FUS cytoplasmic inclusions by disrupting RNA binding, we transfected N2A cells with the EGFP-tagged FUS ALS mutant P525L with or without harboring the acetylation mimicking mutation K315Q/K316Q. The P525L mutation itself caused mis-localization to the cytoplasm as previously reported [107] and ~48% of the P525L mutant-expressing cells showed cytoplasmic inclusions (Figure 1.4D-E). However, only ~11% of cells expressing the P525L/K315Q/K316Q mutant FUS showed cytoplasmic inclusions (Figure 1.4D-E, $p<0.001$). Furthermore, cells expressing EGFP-P525L FUS were treated with the DACi cocktail and the percentage of cells with cytoplasmic inclusions decreased from ~33% in the absence of DACi to ~5% in the presence of DACi (Figure 1.4F-G, $p<0.001$). Thus, the results support that the acetylation of the K315/K316 residues reduces the ability of ALS mutants of FUS to form cytoplasmic inclusions

1.4.6 K510 acetylation is increased in FUS ALS patients

Since the K510 acetylation mimicking mutant significantly increased cytoplasmic mis-localization and inclusion formation (Figure 1.4A-C), we examined the status of K510 acetylation in ALS patients. We first generated a

site-specific antibody against acetylated K510. The rabbit polyclonal antibody specifically detected hyper-acetylated FUS in human HEK293T cells and mouse N2A cells in the presence of DACi, but did not detect any signal in FUS-null N2A cells (Figure 1.6A). Additionally, we transfected FUS-null N2A cells with either empty vector, or WT, K510Q, or K510R FUS with or without DACi cocktail. The antibody detected a strong band in extracts from WT FUS-expressing cells treated with DACi, whereas it did not detect any signal in K510R mutant extract treated with DACi (Figure 1.6B). The acetylation mimicking mutant K510Q produced a very faint band, thus, the antibody is specific for FUS acetylation of K510. We used this acetyl-K510 specific antibody to analyze human skin fibroblast samples from FUS ALS patients and healthy controls. Levels of K510 acetylation were normalized against total FUS. We found ~50% increased levels of acetylated FUS K510 in ALS patients ($p = 0.015$) (Figure 1.7A-B). These results suggest that acetylation of FUS could be associated with pathological characteristics of FUS ALS.

1.4.7 FUS is acetylated by CBP/p300

The CREB-binding protein (CBP) and p300 form a select family of lysine acetyltransferases (KATs) due to their structural and functional similarities [108, 109]. These proteins are responsible for a large portion of the acetylation of histone and non-histone proteins in mammalian cells [109]. FUS has been shown to interact with CBP/p300 [39], and to verify that CBP/p300 acetylates FUS we co-transfected HA-tagged CBP and FLAG-tagged FUS into N2A cells.

After FLAG immunoprecipitation, pan-acetylated lysine antibody showed a strong signal when HA-CBP was present compared to HA-empty vector (Figure 1.8A), indicating that FUS was acetylated by CBP/p300.

We then treated N2A cells with DACi cocktail plus compound A-485, a highly selective CBP/p300 inhibitor [110], at different concentrations for 24h. We detected a significant signal decrease in acetylated-K510 FUS with increasing concentrations of A-485 (Figure 1.8B-C). Interestingly, treatment of 8 μM A-485 did not change the total acetylation as detected by the pan-acetylated lysine antibody while K510 acetylation significantly decreased (Figure 1.8D). The results suggest that endogenous CBP/p300 is a major acetyltransferase at the K510 site of FUS.

1.4.8 FUS is deacetylated by HDAC and Sirtuins lysine deacetylases

Lysine deacetylases are enzymes that play an important role in regulating epigenetic changes that are critical for gene expression. They are classified into different families: HDACs are Zn^{2+}-dependent lysine deacetylases while Sirtuins require nicotinamide adenine dinucleotide (NAD^+) [111]. To identify which family of lysine deacetylases deacetylate FUS, we treated N2A cells with two deacetylase inhibitors for 24h: nicotinamide which inhibits sirtuins [112], and Trichostatin-A (TSA) which inhibits a broad range of HDACs [113]. We first examined the total acetylation by FLAG immunoprecipitation followed by Western blot using a pan-acetylated lysine antibody (Figure 1.8E-F). Cells treated with nicotinamide showed a trend of

increase without statistical significance. Cells treated with TSA showed a significant increase of total FUS acetylation. Cells with both nicotinamide and TSA showed ~8x increase of total FUS acetylation as compared to the control (Figure 1.8E-F). Next, we evaluated the effect of these inhibitors on the K510 site. In cells treated with nicotinamide or TSA alone, we observed a significant increase in FUS-K510 acetylation compared to the vehicle control. In the same fashion, treating with both nicotinamide and TSA together, we detected a ~15x increase in FUS-K510 acetylation (Figure 1.8G-H). This suggests that both HDACs and Sirtuins are able to deacetylate FUS.

We next used co-precipitation assays to identify candidate FUS lysine deacetylases. A set of FLAG-tagged expression constructs for all known human lysine deacetylases (HDACs 1-11 and SIRTs 1-7) were transfected into HEK293T cells, followed by endogenous FUS immunoprecipitation. Among the 11 HDACs and 7 SIRTs (Figure 1.9A-B) tested, only HDAC3 and SIRT7 co-precipitated with the endogenous FUS (Figure 1.8I), suggesting that these lysine deacetylases might be involved in FUS deacetylation. SIRT6 and hnRNPA1 were included as a negative and positive control in the co-precipitation study, respectively (Figure 1.8I). The results in Figure 1.8I are consistent with the results in Figure 1.8E-H that the FUS acetylation level increased in the presence of either HDAC or SIRT inhibitors.

1.5 Discussion

FUS is a DNA/RNA binding protein that belongs to the FET/TET family of proteins, including TAF15 and EWS. FUS mutations have been linked to familial ALS [29] and FUS pathology is also found in a subset of FTD [114]. FUS is a ubiquitously expressed protein that has many molecular functions in the cell. In this study, we found that the biochemical properties of FUS are modulated by acetylation. First, using a mass spectrometry approach, we identified lysine acetylation sites in the RRM domain and C-terminal NLS of FUS. Lysine residues 315 and 316 are located in the KK-loop, which is a structural component of the RRM domain and is involved directly in DNA and RNA binding [57]. The lysine residue 510 is located in the NLS of FUS, a domain that is primarily responsible for importing FUS into the nucleus. Remarkably, mutations in this site (K510E, K510R and K510M) have been reported in familial ALS cases [115-117]. These findings led us to hypothesize that acetylation of lysine at these sites could influence the biochemical function of FUS in the cell.

FUS-NLS domain harbors most of the reported ALS mutations, resulting in aberrant FUS protein mis-localization and aggregation in the cytoplasm [82-85]. The interaction between FUS-NLS and TNPO1 is critical for importing FUS into the nucleus. Structural analysis showed that the positively charged K510 residue interacts with the negatively charged D693 residue of TNPO1 (Figure 1.2J) [81]. It is conceivable that the addition of an acetyl group to K510 could disrupt the interaction between K510 and D693. In

addition, the acetyl group can also cause steric hindrance between the K510 and D693 residues. Thus, K510 acetylation would reduce FUS binding affinity to TNPO1 and increase the cytoplasmic localization of FUS. It was reported that familial ALS patients with the K510R mutation manifest a mild phenotype and long survival after disease onset [117]. Although both arginine and lysine contain positively charged side chains, the Arg side chain is spatially larger than Lys, consistent with the notion that the K510R mutation may disrupt the FUS NLS-TNPO1 interaction to a lesser degree, accounting for mild disease phenotypes. In contrast, FUS K510E ALS patients showed early onset and rapid progression of the disease supporting the concept that a negative charge at position 510 is particularly deleterious [118]. Our previous data showed that K510E has only 1% binding affinity to TNPO1 [81], and it has been shown that a severe disruption of nuclear import could be correlated with a rapid progression of the disease [119]. These results suggest that FUS NLS is sensitive to perturbations at the K510 residue, including mutations or post-translational modifications like acetylation discovered in this study. Indeed, our results showed that FUS acetylation at K510 disrupted the interaction with TNPO1 (Figure 1.3D-E), leading to the cytoplasmic accumulation of FUS and the formation of G3BP1-positive stress granule-like inclusions in the cytoplasm (Figure 1.4A-C, Figure 1.5). Several transgenic mice models have shown that cytoplasmic aggregation and inclusion formation contribute to neurodegeneration [120-122], supporting the gain-of-toxic function hypothesis.

FUS-RRM domain has been reported to bind various nucleic acids, ranging from DNA to G-quadruplex RNA [57]. The K315/K316 motif in FUS-RRM domain exhibits tandem positive charges at the protruding tip of the KK loop and contributes significantly to the electrostatic interaction with the negatively charged nucleic acids. Point mutations K315A/K316A abolished FUS nucleic acid binding [57]. Similarly, our results show that acetylation mimicking mutations in the KK-loop significantly reduce the RNA binding capability of FUS (Figure 1.3A-C), most likely by neutralizing the positive charge of lysine, leading to a decreased affinity to the negatively charged backbone of the nucleic acids [123]. Finally, the attachment of an acetyl group to lysine could cause a steric clash with its interaction partners.

An interesting observation from our immunofluorescence experiments was that transfecting the cells with EGFP-K315Q/K316Q/K510Q caused FUS to mis-localize to the cytoplasm, but the percentage of cells with cytoplasmic inclusions decreased significantly when compared to EGFP-K510Q (Figure 1.4C). These results are in agreement with our previous study that FUS-RNA binding is required for the formation of cytoplasmic inclusions [86]. That study demonstrated that when FUS RNA binding domains were truncated, the formation of inclusions significantly decreased as compared to the R495X FUS ALS mutant. Other work showed that mutating K315 and K316 to Ala residues prevented the formation of cytoplasmic inclusions by the R495X mutant [57]. These data are consistent with the results obtained in this study that introducing the acetylation mimics at the RNA-binding sites into the ALS

mutant P525L (P525L/K315Q/K316Q) significantly reduced the percentage of cells with cytoplasmic inclusions as compared to the P525L mutation alone (Figure 1.4D-E). Consistent with these findings, we showed that treating cells expressing the P525L ALS mutant with deacetylase inhibitor cocktail significantly reduced the percentage of cells with cytoplasmic inclusions (Figure1.4F-G). Our results are relevant to a recent study showing that dynamic multi-valent interaction between FUS and RNA is critical to maintaining the fluidity and function of FUS [124]. FUS mutations that constrain the FUS-RNA interactions lead to higher order complex of ribonucleoprotein aggregates [124]. Finally, RNA binding was also shown to play a critical role in the formation of stress granules and pathological inclusion of TDP-43 [125]. These studies support our conclusion that RNA binding is critical for self-assembly of FUS into cytoplasmic inclusions and that Lys acetylation in the RRM domain can effectively modulate RNA binding and inclusion formation of mutant FUS.

To identify the lysine deacetylases that might regulate FUS acetylation, we first used nicotinamide and TSA to test whether FUS acetylation is susceptible to Sirtuins or HDACs, respectively. Our results showed that total FUS acetylation as well as FUS K510 acetylation can be mediated by both Sirtuins and HDACs (Figure 1.8E-H). It is noted that the effect of nicotinamide alone on the total acetylation level was not statistically significant. It has been reported that the expected inhibitory effect of nicotinamide could be unreliable, in particular, nicotinamide was reported to stimulate the SIRT1 activity [126].

We next examined which specific Sirtuin or HDAC interacted with FUS using immunoprecipitation. We found that SIRT7 and HDAC3 interact with FUS. The FUS-SIRT7 interaction was previously reported in a proteomics study [127] but was not experimentally validated. SIRT7 is a NAD+-dependent deacetylase that acts on histone and non-histone proteins [128] and it is localized in the nucleus, but primarily in the nucleolus [129]. Moreover, we identified a novel interaction between HDAC3 and FUS. HDAC3 is a Zn^{2+}-dependent class-I HDAC that has been reported to shuttle between the nucleus and the cytoplasm [130]. An interaction between FUS and HDAC1 was reported previously [38], however we could not confirm this interaction under our experimental conditions. Future studies of genetically reducing HDAC3 and SIRT7 are needed to confirm that they indeed deacetylate FUS.

We also demonstrated that endogenous CBP/p300 is a major acetyltransferase for FUS, particularly the K510 site (Figure 1.8A-D). The CBP/p300 inhibitor A-485 [110] significantly decreased FUS K510 acetylation in a dose-dependent manner in N2A cells (Figure 1.8B-C), however the total FUS acetylation did not show significant change, suggesting that the acetylation of the specific K510 site is more sensitive to CBP/p300 inhibitor. Reports show that CBP/p300 is localized to the nucleus [131] and is responsible for acetylation of over two-thirds of the lysine acetylation sites. Overall, the process of FUS acetylation/deacetylation appears to be very dynamic, and it is likely that other acetyltransferase enzymes play a role in FUS acetylation at other sites such as K315/K316. The functional

consequence of inhibiting the specific acetyltransferases (e.g. CBP/p300) and deacetylases (e.g. SIRT7 and HDAC3) on the subcellular localization and inclusion formation of FUS remains to be determined in future studies.

To our knowledge, lysine acetylation of human FUS has not been reported previously. A proteomics study reported the acetylation of K502 in rat FUS and K640 in rat EWS, the equivalent of K510 in human FUS. The acetylation of the K263 residue in rat TAF15 was also reported, which corresponds to K316 in human FUS [132]. Although the acetylation of those residues was not further validated and their functional relevance was not studied, the proteomic identification of acetylation of these conserved residues in the FET family proteins across species is supportive of the importance of our findings demonstrating acetylation of K315, K316 and K510 residues. Our mutagenesis and pharmacological studies demonstrate that acetylation of these residues bestowed a profound impact on FUS subcellular localization as well as the formation of stress granule-like cytoplasmic inclusions.

More interestingly, a higher K510 acetylation level was observed in familial FUS ALS patients as compared to healthy controls (Figure 1.7A-B), suggesting that acetylation could play a role in disease pathogenesis and/or serve as a molecular hallmark of the disease. Particularly, K510 acetylation increased the cytoplasmic inclusions (Figure 1.4A-C). Based on all results in this study, a working model is proposed to illustrate the role of FUS acetylation (Figure 1.10). Acetylation of K315/K316 disrupts the RNA binding but does not interfere with the TNPO1-mediated nuclear import of FUS. In contrast,

acetylation of K510 disrupts the interaction with TNPO1 and promotes the formation of cytoplasmic inclusions. When all three lysine residues are acetylated, FUS accumulates in the cytoplasm but forms less inclusions due to impaired RNA binding. We propose this process to be dynamic and the three forms of FUS acetylation can be present simultaneously. For ALS-linked mutations, mutant FUS shows reduced TNPO1 binding, impaired nuclear import, and cytoplasmic accumulation. Moreover, mutant FUS shows a higher level of K510 acetylation in ALS patients, facilitating the formation of cytoplasmic inclusions. Future studies are needed to test whether CBP/p300 inhibitors can mitigate mutant FUS mediated disease pathology by suppressing the K510 acetylation.

The pathologic inclusions are a clinical characteristic of mutant FUS mediated ALS/FTD. Studies show that different types of stress granules can form in a G3BP1-dependent [133] or independent [134] fashion, and can generate different functional outcomes [135]. The role of acetylation of FUS in stress granules and the functional consequence of such acetylation-dependent granules remains to be determined in future studies. In addition, a recent study reported that nuclear-to-cytoplasmic mis-localization of FUS without aggregation in the cytoplasm occurred in different models of FUS ALS [136]. We propose that pharmacologically modulating acetylation of FUS can prevent protein mis-localization or formation of pathological inclusions, providing new targets for therapeutic treatments against FUS-mediated ALS/FTD and other neurodegenerative diseases.

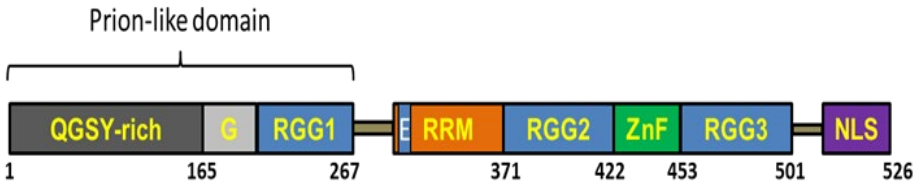

Figure 1.1 The functional domains of FUS

QGSY-rich, Glutamine/Glycine/Serine/Tyrosine rich domain; G, Glycine rich domain; RGG, Arginine/Glycine/Glycine rich domain; E, Nuclear export signal; RRM, RNA recognition motif; ZnF, Zinc finger domain; NLS, Nuclear Localization Signal

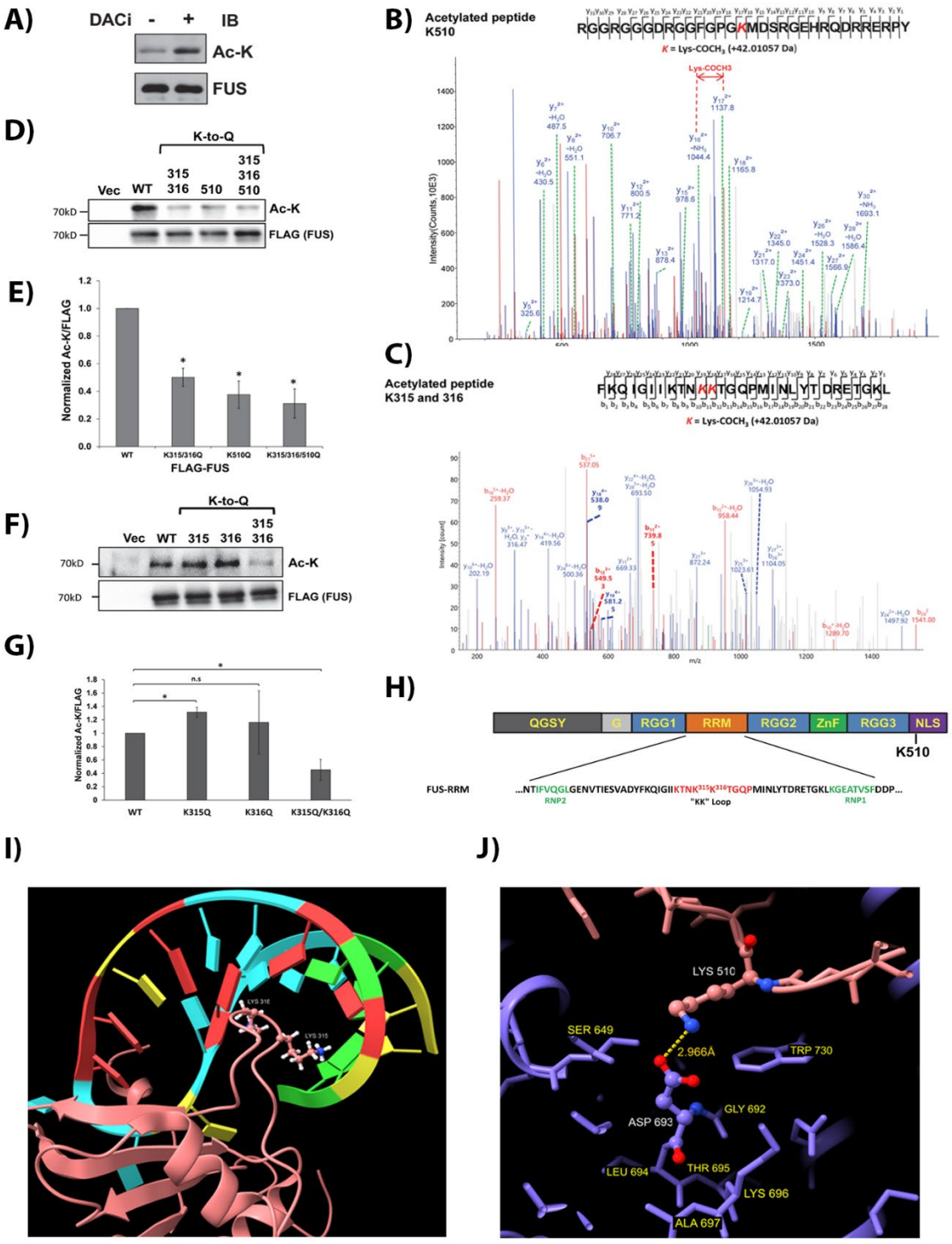

Figure 1.2 FUS is acetylated

(A) Endogenous FUS immunoprecipitation from N2A cells treated with deacetylase inhibitor cocktail (DACi) (nicotinamide (30 mM), sodium butyrate (50 mM) and Trichostatin-A (3 μM)) for 6h. Immunoblotting was performed using the indicated antibodies. (B) Mass spectrometric identification of the acetylated FUS peptide RGGRGGGDRGGFGPGK^{510}MDSRGEHRQDRRERPY. (C) The MS/MS spectrum of the di-acetylated peptide ^{305}FKQIGIIKTNKKTGQPMINLYTDRETGKL^{333}. The series of matched b and y ions are shown in blue and red, respectively. The b11, b12, y18 and y19 ions supporting the K315 and K316 acetylation sites are labeled in bold. (D) 3×FLAG-tagged WT, K315, K316Q, K315Q/K316Q or FLAG vector control were transfected into N2A cells. After 24h, cells were treated with deacetylase inhibitor cocktail for 6h, followed by FLAG immunoprecipitation and immunoblotting with the indicated antibodies. (E) Quantification of FUS acetylation from three independent experiments ± SD. Student's *t*-test was performed for individual comparisons against WT. *, p ≤0.05. (F) 3×FLAG-tagged WT, K315Q/K316Q, K510Q, K315Q/K316Q/K510Q or FLAG vector control were transfected into HEK293T cells. After 24h, cells were treated with deacetylase inhibitor cocktail for 6h, followed by FLAG immunoprecipitation and immunoblotting with the indicated antibodies. (G) Quantification of FUS acetylation from three independent experiments ± SD. Student's *t*-test was performed for individual comparisons against WT. *, p ≤0.05. (H) The domain structure of FUS showing the RRM domain sequence and acetylation sites. (I)

NMR solution structure of FUS RRM domain (pink) showing K315 and K316 in the KK-loop bound to stem-loop RNA (Protein Data Bank entry 6GBM). (J) Crystal structure of TNPO1/FUS-NLS (Protein Data Bank entry 4FQ3) illustrating the FUS-NLS domain (pink) K510 adjacent to TNPO1 (purple) D693. I-J were generated using the UCSF ChimeraX software.

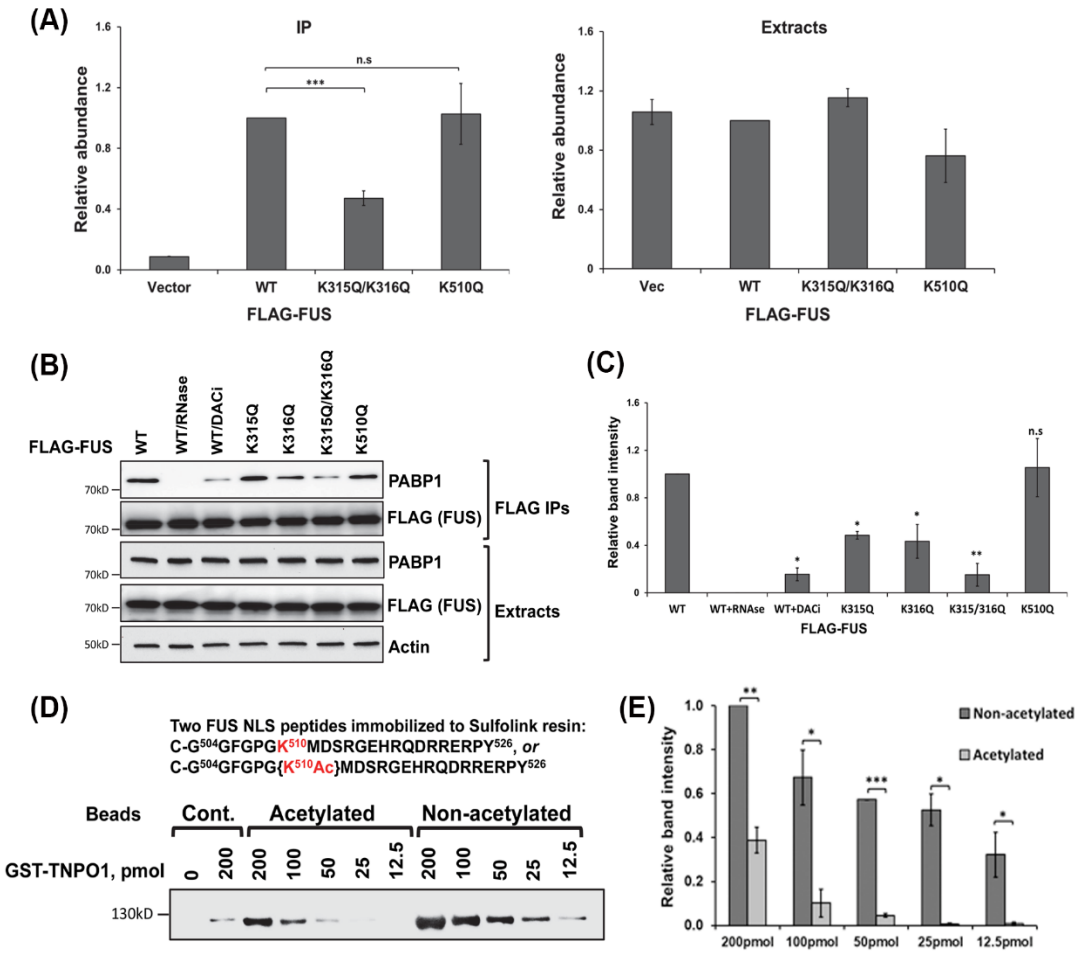

Figure 1.3. Acetylation of FUS impairs RNA binding and the FUS NLS-TNPO1 interaction

(A) 3×FLAG-tagged FUS constructs were transfected into N2A cells. After 48h, cells were lysed, and FLAG-IP followed by reverse transcription and quantitative PCR against FUS pre-mRNA surrounding exon 7 was performed. FUS mRNA was normalized with the FLAG levels in the FLAG-FUS immunoprecipitations and with the Rpl13a mRNA levels in the total extracts. Averages of three independent experiments are shown, ± SD. Student's *t*-test was performed for individual comparisons against WT. (B) The indicated

3×FLAG-tagged FUS constructs were transfected into N2A cells and treated with DACi cocktail where indicated. FLAG immunoprecipitation was performed 48h after transfection with the inclusion of RNase cocktail as indicated, followed by immunoblotting with the indicated antibodies. (C) Quantification of (B) from three independent experiments. Student's *t*-test was performed for individual comparisons against WT. Significance: *, p ≤0.05; **, p≤0.005; ***, p ≤0.001; n.s: not significant. (D) *In vitro* TNPO1 pulldown with Sulfolink-immobilized acetylated or non-acetylated FUS-NLS peptides. Different amounts of GST-TNPO1 were incubated with FUS-NLS peptide immobilized on the beads at 4°C for 3 hours. The amount of TNPO1 pulled down with FUS-NLS peptides were evaluated by Western blot. (E) Quantification of (D) from three independent experiments, ± SD. Student's *t*-test was performed comparing the band intensities of non-acetylated and acetylated peptide pulldowns at the same concentration. Significance: *, p ≤0.05; **, p≤0.005; ***, p≤0.001; n.s: not significant

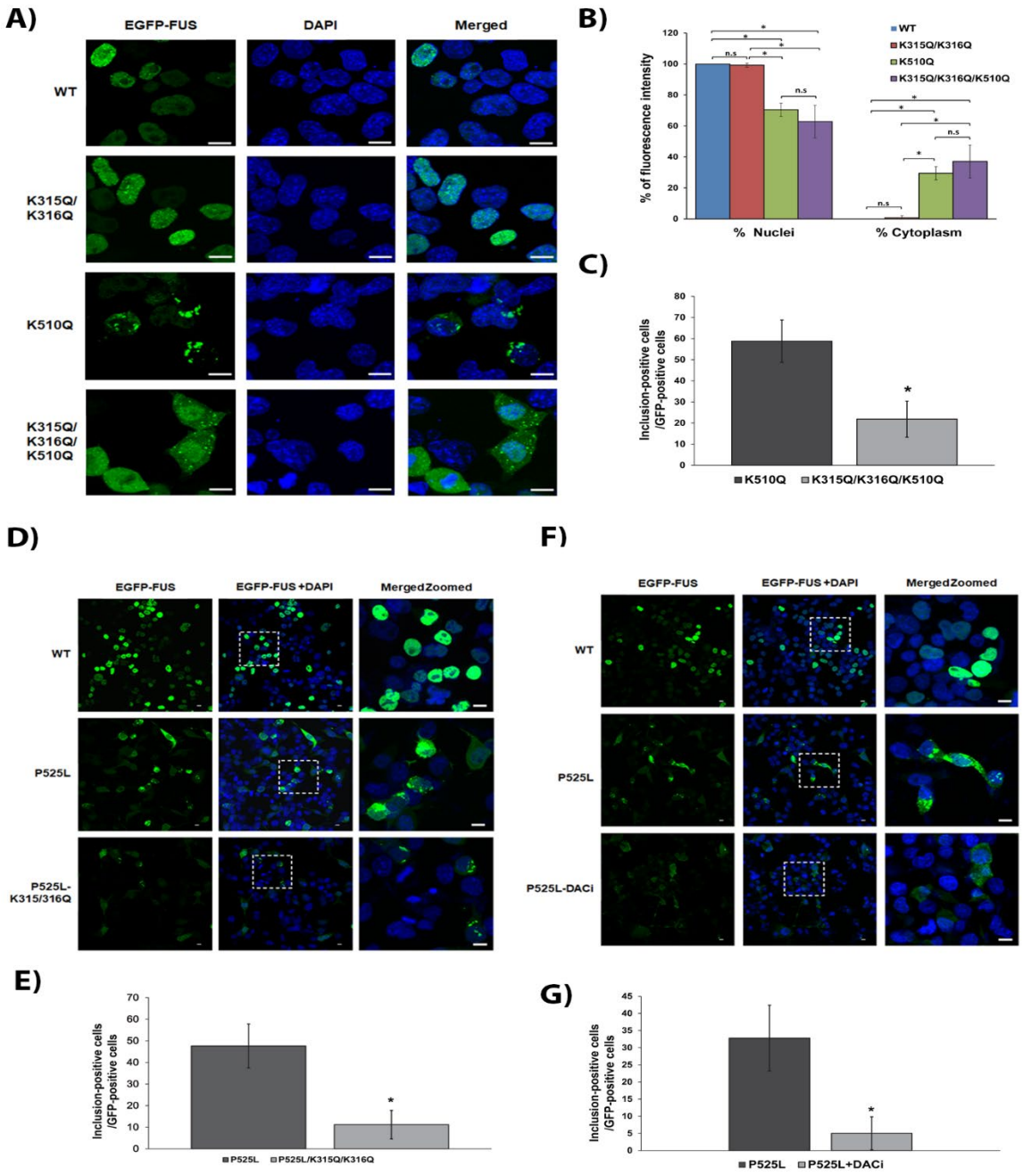

Figure 1.4. The effect of FUS acetylation on cellular localization and stress granule formation.

(A) N2A cells were transfected with EGFP-tagged WT, K315Q/K316Q, K510Q and K315Q/K316Q/K510Q FUS. The nuclei were visualized with DAPI.

Samples were examined by confocal microscopy. Scale bars, 10μm. (B) Quantification of nuclear and cytoplasmic EGFP-tagged FUS intensity ± SD (n>200 cells) using ImageJ scripts. One Way Anova was performed to determine statistical significance. *p ≤0.05; **p≤0.005; ***p≤0.001; n.s: not significant. (C) The percentage of EGFP-positive cells with cytoplasmic inclusions (n > 100 cells). * p ≤0.001. (D) N2A cells were transfected with EGFP-tagged WT, P525L, and P525L/K315Q/K316Q FUS. The nuclei were visualized with DAPI. Samples were examined by confocal microscopy. Scale bars, 10μm. (E) The percentage of EGFP-positive cells with cytoplasmic inclusions (n > 100 cells). * p ≤0.001. (F) N2A cells were transfected with EGFP-tagged WT or P525L FUS. One set of P525L transfected cells were treated with deacetylase inhibitor (DACi) cocktail (30 mM nicotinamide, 50 mM sodium butyrate and 3 μM Trichostatin-A). The nuclei were visualized with DAPI. Samples were examined by confocal microscopy. Scale bars, 10μm. (G) The percentage of EGFP-positive cells with cytoplasmic inclusions (n > 100 cells). * p ≤0.001.

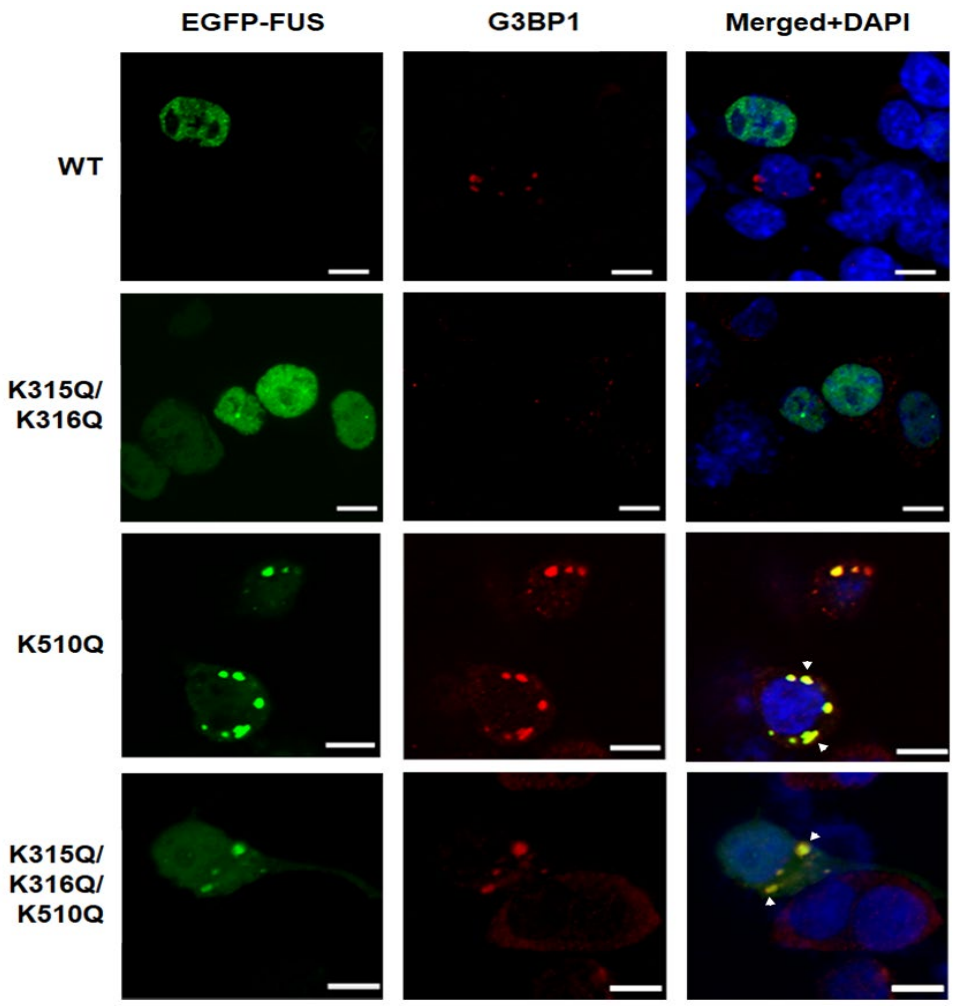

Figure 1.5. FUS acetylation mimicking mutant inclusions co-localize with the stress granule marker G3BP1.

Co-localization of endogenous G3BP1 with EGFP-tagged FUS in N2A cells. The co-localization of FUS and G3BP1 is shown by arrows. Scale bars, 10μm.

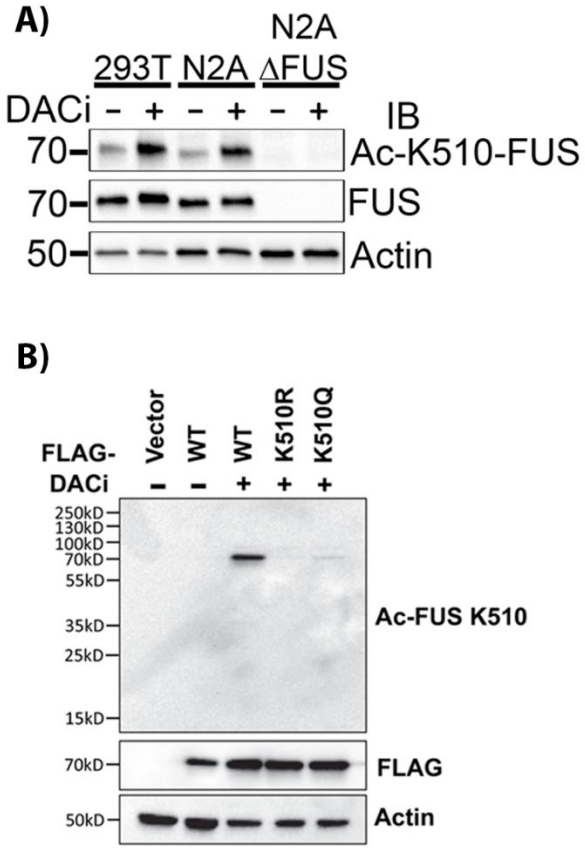

Figure 1.6. Characterization of the anti-K510-acetylated FUS antibody.

(A) HEK293T, N2A, and N2A-ΔFUS (FUS knockout) cells were treated with deacetylase inhibitor (DACi) cocktail (30 mM nicotinamide, 50 mM sodium butyrate and 3 μM Trichostatin-A), as indicated. Immunoblotting was performed using the indicated antibodies. (B) N2A ΔFUS cells were transfected with 3×FLAG-tagged WT, K510R, or K510Q FUS or vector control with or without DACi cocktail as indicated. Immunoblotting was performed using the indicated antibodies.

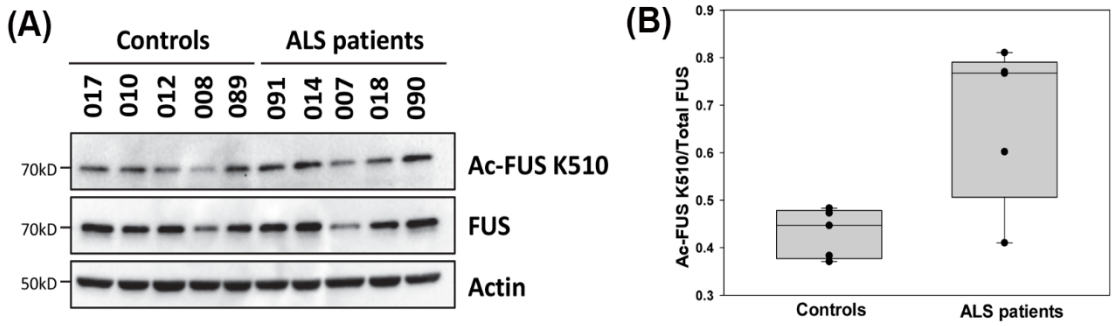

Figure 1.7. K510 acetylation is increased in familiar FUS ALS.

(A) FUS-K510 acetylation levels in ALS patients with R521G or P525R FUS mutations versus control subjects. Immunoblotting was performed using the indicated antibodies. (B) Quantification of FUS-K510 acetylation normalized against total FUS levels. Error bars represent SD between individuals. One Way Anova was performed to determine statistical significance. *, $p \leq 0.05$.

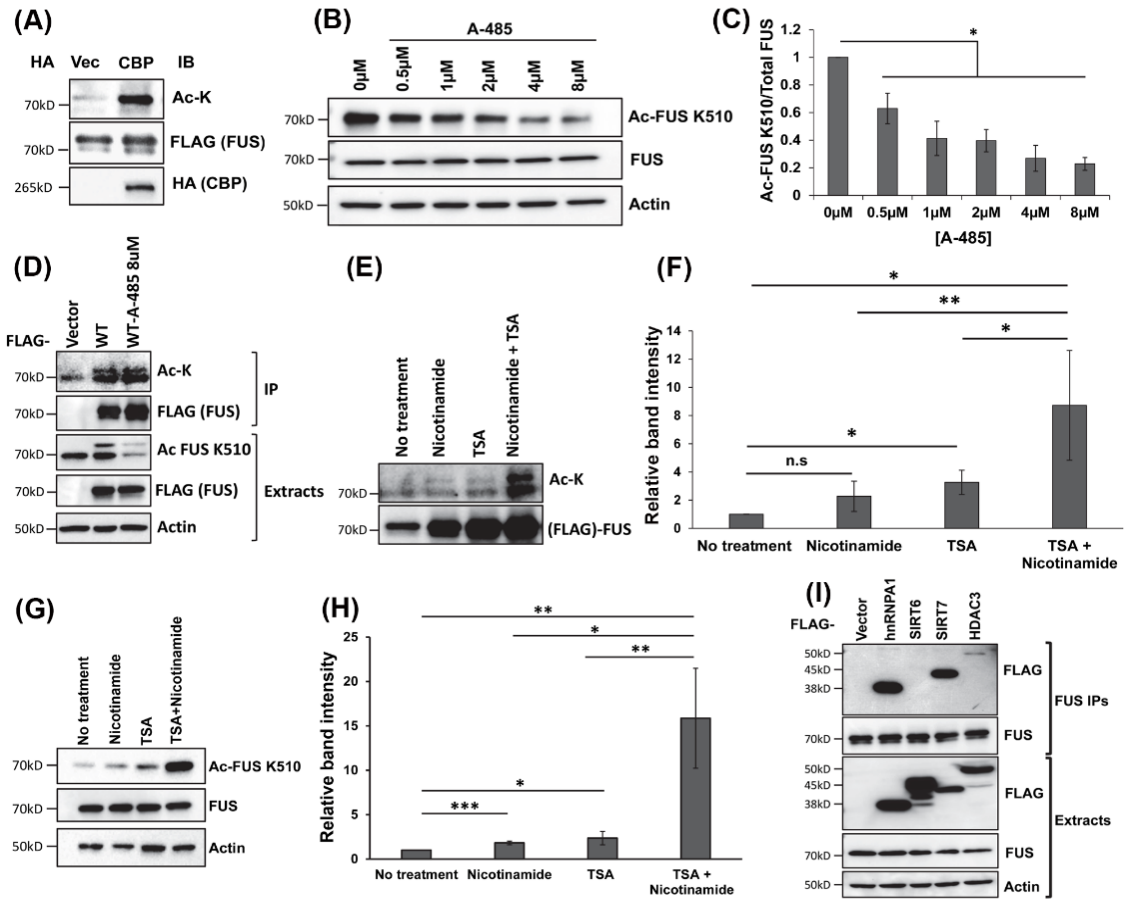

Figure 1.8. The regulators of FUS acetylation

(A) 3×FLAG-FUS or 3×FLAG-vector was co-transfected with HA-CBP or HA-vector into N2A cells. FLAG immunoprecipitation was performed, followed by immunoblotting using the indicated antibodies. (B) N2A cells were treated with different concentrations of the CBP/p300 inhibitor A-485 in the presence of DACi cocktail. Immunoblotting was performed using the indicated antibodies (C) Quantification of (B), from three independent experiments ± SD. Student's t-test was performed for individual comparisons against no

treatment. *p ≤0.05. (D) N2A cells were transfected with FLAG-vector or FLAG-FUS and treated with CBP/p300 inhibitor A-485 (8μM) for 16h in the presence of DACi cocktail. FLAG immunoprecipitation was performed, followed by immunoblotting with the indicated antibodies. (E) N2A cells were transfected with FLAG-FUS and treated with 30 mM nicotinamide and/or 3 μM Trichostatin-A for 6h. FLAG immunoprecipitation was performed followed by immunoblotting with the indicated antibodies. (F) Quantification of (E), from three independent experiments ± SD. (0μM). *p ≤0.05. One-way Anova was performed to determine statistical significance. *, p ≤0.05; **, p≤0.005; (G) N2A cells were treated with 30 mM nicotinamide and/or 3 μM Trichostatin-A for 6h. Cells were harvested and lysed after treatment and immunoblotting was performed using the indicated antibodies. (H) Quantification of (G), from three independent experiments ± SD. One-way Anova was performed to determine statistical significance. *, p ≤0.05; **, p≤0.005; ***, p≤0.001; n.s,: not significant. (I) HEK293T cells were transfected with 3×FLAG-empty vector, 3×FLAG-SIRTs 6-7, 3×FLAG-HDAC3, and 3×FLAG-ROA1 (hnRNPA1) used as a positive control. After 48h, endogenous FUS immunoprecipitation was performed, followed by immunoblotting using the indicated antibodies.

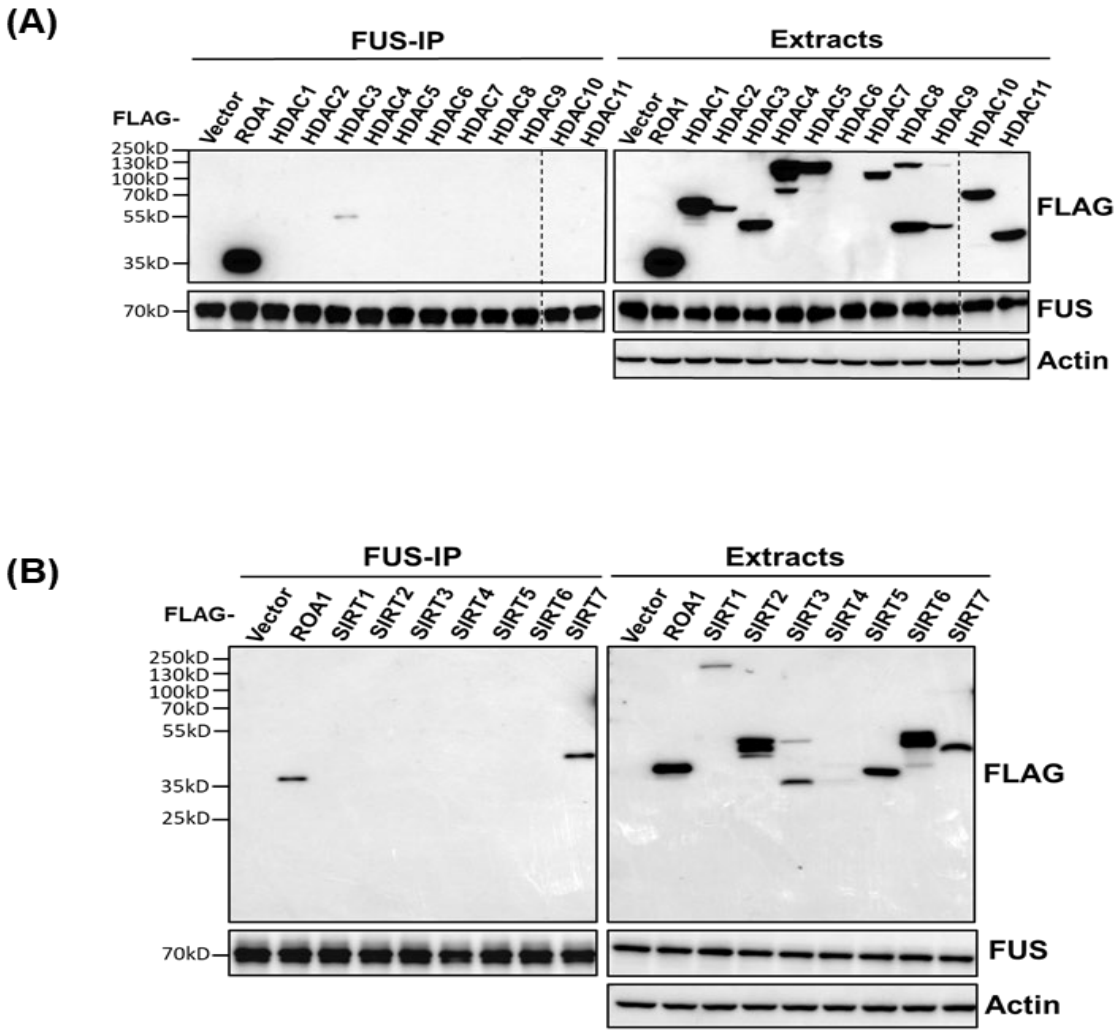

Figure 1.9. Interaction screening of FUS with lysine deacetylases.

(A) HEK293T cells were transfected with 3×FLAG-HDAC 1-11 (A) or 3×FLAG SIRT 1-7 (B). After 48hrs, endogenous FUS immunoprecipitation was performed, followed by immunoblotting using the indicated antibodies. The 3×FLAG-ROA1 (hnRNPA1) construct was used as a positive control.

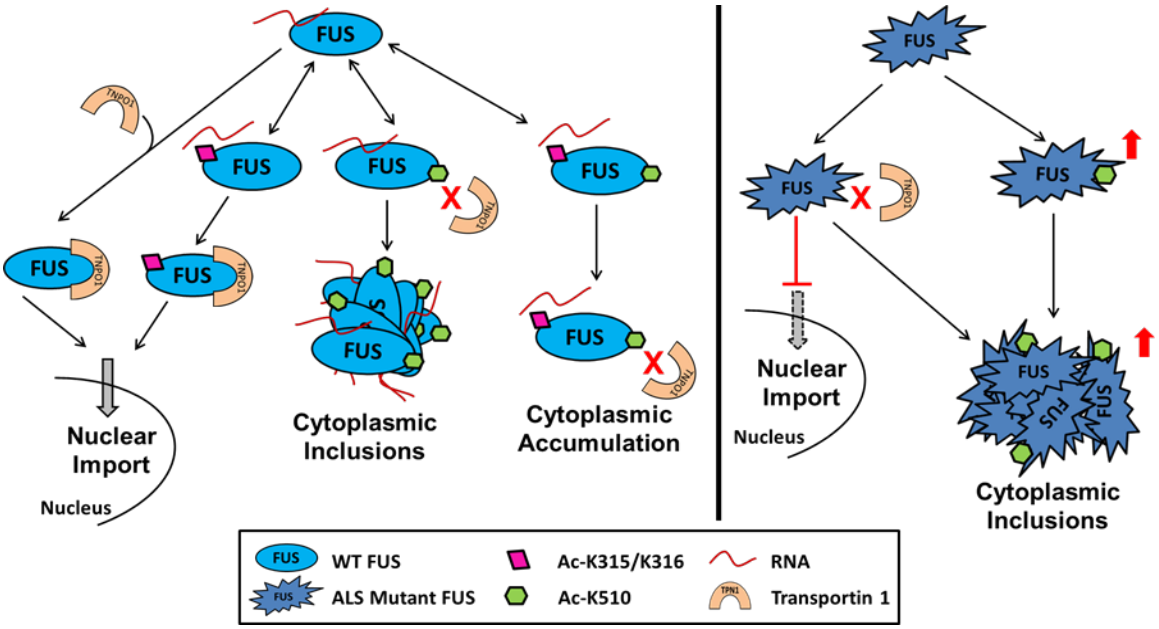

Figure 1.10. Proposed model of the role of FUS acetylation in the modulation of FUS subcellular localization and inclusion formation

Acetylation of K315/K316 disrupts the RNA binding but does not interfere with the TNPO1-mediated nuclear import of FUS. In contrast, acetylation of K510 disrupts the interaction with TNPO1 and promotes the formation of cytoplasmic inclusions. When all three lysine residues are acetylated, FUS accumulates in the cytoplasm but forms less inclusions due to impaired RNA binding. We propose this process to be dynamic and the three forms of FUS acetylation can be present simultaneously. For ALS-linked mutations, mutant FUS shows reduced TNPO1 binding, impaired nuclear import, and cytoplasmic accumulation.

CHAPTER 2. FUS REGULATES THE TRANSCRIPTION OF GENES CRITICAL TO AUTOPHAGY

2.1 Abstract

FUS/TLS (Fused in Sarcoma/Translocated in Liposarcoma) is a ubiquitously expressed RNA-binding protein that has been reported to play different roles in the cell. Mutations in FUS have been linked to a subset of familial cases of the neurodegenerative disease amyotrophic lateral sclerosis (ALS). FUS is mainly localized in the nucleus, although it shuttles between the nucleus and the cytoplasm to transport RNAs, and it is also present in the cytoplasm of neuronal cells. [29]. Mutations in the nuclear localization sequence (NLS) of FUS, causes its mis-localization to the cytoplasm where it forms inclusions that co-localize with stress granule proteins. Stress granules are dynamic complexes that are form by proteins, ribosomes and RNAs, and they can be degraded by the autophagy pathway. It has been shown that stress granules containing mutant FUS co-localize with autophagosomes, however the role of FUS in this pathway needs to be elucidated. In this study we found that KO FUS cells had a decreased basal autophagy flux. Treatment with Bafilomycin A1 and rapamycin showed that KO FUS cells were not able to induce autophagy as efficiently as WT cells, suggesting that the initial phases of the autophagy pathway are affected in FUS KO cells. We found that FIP200, ATG16L1, and ATG12 mRNA and protein levels were significantly lower in FUS KO cells. Overexpressing FUS in FUS KO cells was able to rescue gene and protein expression levels of FIP200 and ATG16L1. Our findings demonstrate a novel role of FUS in the autophagy pathway, by

regulating the transcription of genes involved in the initial stages of autophagy, such as initiation and phagophore elongation.

2.2 Introduction

2.2.1 FUS in cytoplasmic inclusions and stress granules dynamics

Many of the familial ALS-related FUS mutations are localized in the C-terminal NLS, causing mis-localization of FUS to the cytoplasm where it forms inclusions that co-localize with stress granule proteins [82-85]. Cytoplasmic aggregates are a hallmark of neurodegenerative diseases, with neurofibrillary tangles (NFTs) in Alzheimer's disease (AD) [137], Lewi bodies in Parkinson's disease (PD), or inclusion bodies in ALS tissue samples [138]. In fact, all sporadic and familial non-SOD1 ALS patients, display FUS inclusions in the spinal cord. These inclusions are also immunoreactive to TDP43, p62, and ubiquitin [139].

Moreover, protein aggregation can be induced after cellular stress [140]. Stress granules are dynamic membrane-less cytoplasmic complexes of proteins, ribosomes, and RNAs that form under oxidative stress, hyperosmolar stress, or heat shock [141]. The precise role of stress granules remains to be fully understood, however it has been suggested that they can serve as storage sites for further mRNA translation or degradation [142]. Several studies have reported that mutant FUS inclusions, co-localized with stress granules markers [82, 83, 143-145]. A study showed that cells expressing

ALS-linked FUS mutations form cytoplasmic inclusions that co-localize with stress-granule proteins such as PABP1, TIA-1, G3BP1 [83, 146]. However, FUS is not required for stress granule assembly [145]. The previous evidence shows that FUS is involved in stress granule dynamics, and that alterations in this process by FUS mutations can lead to aberrant aggregation with other RNA-binding proteins and RNAs. Protein turn-over pathways, particularly autophagy, help clear pathogenic aggregates from the cells and maintain protein homeostasis.

2.2.2 Autophagy pathway

There are two major protein degradation pathways: the ubiquitin-proteasome system (UPS) and the autophagy pathway. The UPS is responsible for the degradation of short-lived soluble proteins, while the autophagy pathway can degrade misfolded proteins and whole organelles [147] (The autophagy pathway is illustrated in Figure 2.1).

mTOR is a well-known regulator of autophagy that acts as an inhibitor of the pathway when the cells are under nutrient-rich conditions, by preventing the formation of the autophagy initiation complex (ULK1-ATG13-FIP200). When starvation conditions hit the cells, or cells are treated with rapamycin, mTOR is inhibited and autophagy starts with activation of the initiation complex and formation of the phagophore [148].

Subsequently, elongation of the phagophore is facilitated by two ubiquitin-like systems: ATG12:ATG5:ATG16L system and PE-LC3 system. These two processes are governed by ATG (AuTophaGy-related proteins). The first complex receives autophagy initiation signals, and localizes to the autophagy isolation membrane for elongation [149]. A sequence of events that resemble the ubiquitin-proteasome pathway are involved in this step. First, Atg12 binds Atg5 through an E1-like enzyme Atg7. Then, Atg12-Atg5 conjugates with Atg16L1 through the reaction with E2-like enzyme Atg10 [149], resulting in the ATG12:ATG5:ATG16L system.

Similarly, PE-Atg8 system is in charge of Atg8 processing. Atg8 (LC3) is synthesized with an extra C-terminus sequence that is removed by Atg4. This process helps to expose the glycine residue of Atg8 (LC3-I), that is required for the next steps of the reaction. Then, Atg7 and Atg3 activate Atg8, in order to attach phosphatidylethanolamine (PE) to the Atg8-C-terminal glycine (LC3-II). PE is necessary for the attachment of this protein to the phagophore membrane. Finally, Atg12-Atg5-Atg16L1 and Atg8-PE function synergistically to promote autophagosome elongation [150].

When the phagophore elongates and encloses the targeted misfolded proteins, or damaged organelles, it forms a double-membraned autophagosome [151]. Afterwards, Atg12-Atg5-Atg16L disassembles from the membrane, but LC3-II remains attached in the autophagosome membrane until the latest steps of the pathway. Thus, LC3-II is a good marker to evaluate autophagy flux [152].

Finally, the mature autophagosome fuses with the lysosome, and its contents are degraded by acidic proteases. Subsequently, the amino acids and other by-products of the degradation are recycled back into the cytoplasm, where they can be re-used for cell metabolism [153].

2.2.3 Autophagy and ALS

Neurons are post-mitotic cells that are highly susceptible to protein aggregation, thus defects in protein homeostasis are particularly detrimental for neurons. The balance between protein folding, assembly and degradation is necessary for the normal function of the cells [154] This balance is maintained by several factors, including proteolytic machinery and protein chaperones [155, 156].

Defects in the autophagy pathway have been reported in neurodegenerative diseases such Alzheimer's disease and ALS [157-159]. A pathological characteristic of ALS is the accumulation of insoluble protein aggregates in the cytoplasm of motor neurons [154]. Under normal conditions, when protein aggregates form, cells have quality control mechanisms to degrade misfolded proteins, to prevent protein aggregation, or to clean up aggregates that have already formed [147]. However, under pathological conditions, cytoplasmic aggregates result from misfolded proteins that lose their native conformations and fail to be cleared from the cell by the ubiquitin-proteasome pathway or autophagy [147]. The common phenotype of protein

aggregation in neurodegenerative diseases suggests that the mechanisms to maintain protein homeostasis are disrupted and that cytoplasmic aggregates become aberrant and might contribute to cytotoxicity [147].

Some studies have found evidence that autophagy dysregulation might be associated to ALS. For instance, a study analyzed post-mortem samples from sporadic ALS patients and found that cytoplasmic inclusions in motor neurons were immunoreactive to p62 and LC3-II [160]. In another study, p62-positive inclusions were found in iPSCs derived from patients with C9orf72 expansion [161]. Interestingly, when autophagy was inhibited by chloroquine treatment, cell viability decreased in these cells. In another study involving iPSCs derived from ALS/FTLD patients with C9orf72 haploinsufficiency, LC3-II autophagy flux as significantly reduced [162]. Together, these studies suggest that autophagy is essential for maintaining protein homeostasis in neuronal cells.

In a performed in SOD1 G93A mice, an autophagy inducer lithium was found to delay the disease onset and increase the life span. The neuroprotective effects were coupled with the autophagy activation and a larger number of mitochondria in motor neurons [163]. Interestingly, a pilot study in 44 ALS patients with no placebo-controls showed that patients treated with lithium carbonate survived until the end of the study while 29% of patients treated with riluzole died by the same time [163]. These results suggest that autophagy induction might provide a protective effect for neurodegeneration. However, a multicentre, randomised, double-blind, placebo-controlled clinical

trial found that, although there were no safety concerns, there was no evidence of benefit of lithium on survival in patients with ALS (cite: Lancet Neurol. 2013 Apr; 12(4): 339–345.). Thus, the clinical efficacy of the autophagy inducer lithium in ALS remains unclear.

A study showed that autophagy deficient mouse embryonic fibroblasts (MEFs) showed an increase in mutant FUS-positive stress granules. In addition, stress granules that contain mutant FUS co-localize with autophagosomes [157]. Furthermore, induction of autophagy by rapamycin treatment in mutants FUS cells, reduced stress granule formation and cell death in neurons [157] . These results suggest that the autophagy pathway is important for clearance of mutant FUS cytoplasmic inclusions. In another study, post-mortem tissue from sporadic adult-onset ALS showed basophilic inclusions that were immunoreactive for p62, LC3, and FUS [164]. A recent study showed that mutant FUS disrupts autophagy by affecting the formation of the omegasome [165], a membrane derived from the ER, and sites where phagophores form [166]. Together, these studies indicate that autophagy is an important mechanism involved in neurodegeneration, and dysregulation of this pathway may cause negative effects in motor neurons, leading to diseases such as ALS.

2.2.4 Study rationale

In summary, neurodegenerative diseases are characterized for toxic protein aggregation in the cytoplasm of neuronal cells. Since these are post-

mitotic cells that do not undergo cell division, they are susceptible to defects in protein homeostasis. A few studies have shown a connection between FUS and the autophagy pathway. However, the specific function of FUS in this pathway remain to be studied. Here, we asked whether mutant FUS disrupts autophagy flux, and whether loss of FUS affects the expression of proteins involved in various stages of autophagy.

2.3 Materials and Methods

2.3.1 Cell culture and transfection

N2A, FUS KO N2A, and NSC34 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, D5796) with 10% fetal bovine serum, penicillin-streptomycin, and amphotericin B at 37°C in 5% CO_2/95% air with humidification. The pCMV10-3×FLAG-FUS plasmids, were generated as reported [83]. Cells were treated with bafilomycin A1 (200nM) for 6 hours, Rapamycin (2μM) for 4h, or MG-132 (5μM) for 16 hours. FUS mutations were introduced using the QuickChange II Site-Directed Mutagenesis Kit (Agilent). N2A, FUS KO N2A and NSC34 cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific, 11668). siRNA-FUS (Santa Cruz, sc-40563) oligonucleotides were reversed-transfected into N2A and NSC34 cells at a final concentration of 1μM using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, 13778100).

2.3.2 Generation of FUS KO (ΔFUS) N2A cells.

The FUS knockout cells were generated by employing CRISPR technology. N2A cells were transfected with FUS double nickase CRISPR plasmid (Santa Cruz Biotechnology, sc-433326-NIC) following the manufacturer's instructions. Clonal cell lines were isolated with serial dilution, and the FUS status of the clones was determined with immunoblotting.

2.3.3 Generation of the FLAG-FUS knock-in cells lines

FLAG-tagged FUS WT was transfected into FUS KO N2A cells using Lipofectamine 2000 as described above. Selection of stable cells with FLAG-FUS WT integration was performed with G-418 (Millipore Sigma G418-RO). Clonal cells lines were isolated with serial dilution, and the FUS expression levels were compared to endogenous FUS in N2A cells by western blot, using anti-FUS and anti-FLAG antibodies.

2.3.4 Western blot analysis

Cells were lysed with 1x RIPA buffer (Millipore Sigma, 20-188) supplemented with protease inhibitor cocktail (Millipore Sigma, P8340, 1:500) and sodium orthovanadate (1 mM). Lysates were homogenized by sonication and centrifuged at 1,000g for 15 minutes at 4°C. Supernatant was collected and protein concentration was determined using a colorimetric Protein Assay

Dye Reagent Concentrate (BIO-RAD, 5000006,). 6x SDS buffer sample was added to samples containing equal amounts of protein and heated for 5 min at 95ºC. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Pall, 66485). The membranes were blocked with 5% non-fat dry milk in TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween-20) or bovine serum albumin (BSA) for 1 hour.

2.3.5 Antibodies

The antibodies used include mouse anti-FUS (Santa Cruz, sc-47711), mouse monoclonal ANTI-FLAG® M2-Peroxidase (HRP) (Millipore Sigma, A8592), rabbit anti-β-actin (Cell Signaling 8457). LC3 (Millipore Sigma L8918), Mouse anti-SQSTM-p62 (H00008878-M01), Autophagy Induction (ULK1 Complex) Antibody Sampler Kit (Cell Signaling 46486,) containing ULK1 (D8H5) Rabbit mAb (Cat #8054), Atg13 (D4P1K) Rabbit mAb (Cat# 13273), FIP200 (D10D11) Rabbit mAb (Cat#12436), Atg101 (E1Z4W) Rabbit mAb (Cat#13492), Phospho-ULK1 (Ser757) (D7O6U) Rabbit mAb (Cat#14202), Phospho-ULK1 (Ser555) (D1H4) Rabbit mAb (Cat#5869). Autophagy Antibody Sampler Kit (Cell Signaling, 4445) containing Beclin-1 (D40C5) Rabbit mAb (Cat#3495), Atg5 (D5F5U) Rabbit mAb (Cat#12994), Atg12 (D88H11) Rabbit mAb (Cat#4180), Atg16L1 (D6D5) Rabbit mAb (Cat#8089), Atg7 (D12B11) Rabbit mAb (Cat#8558), Atg3 Antibody (Cat#3415).

2.3.6 TaqMan qPCR Assay

RNA isolation was performed using Aurum Total RNA Mini Kit (BIO-RAD, 732-6820) following the manufacturer's instructions. 1µg of isolated RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, 18080). The resulting cDNA was subjected to Real-time PCR. Briefly, FAM-labeled Taqman probes for autophagy related genes and controls were obtained from the TaqMan Gene expression assay library from ThermoFisher Scientific. TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific, 4444556, Thermo Fisher Scientific) was prepared following manufacturer's instructions. The assay IDs for the FAM-labelled probes analyzed are: Mm00456545_m1 (Rbcc1, FIP200), Mm00509659_m1 (Atg101), Mm00512209_m1 (Atg7), Mm00471287_m1 (Atg3), Mm00503201_m1 (Atg12), Mm00513085_m1 (Atg16L1), Mm01612987_g1 (Rpl13a), Mm99999915_g1 (GAPDH), Mm00607939_s1 (Actin). The qPCR results were analyzed using the $\Delta\Delta$CT method.

2.3.7 Statistical analysis

Lane quantification for Western blot bands was performed using Image Lab software by BioRad. Statistical analysis was calculated with SigmaPlot 14.0 software. Band intensities were calculated and comparison between groups was performed using Anova with post hoc Tuckey HSD test. Student's

t test was used to determine statistical significance between two groups. Experiments were not blinded. All experiments were done in triplicates.

2.4 Results

2.4.1 ALS-linked mutant FUS did not affect autophagy

First, we tested whether the ALS-linked FUS mutations would affect the levels of basal autophagy. FUS KO N2A cells were transfected with FLAG-tagged WT, R495X, R521G, or P515L FUS. The levels of autophagy markers LC3-I, LC3-II, and p62 were subsequently analyzed by Western blot. To our surprise, the LC3-II/LC3-I ratios and the p62 level did not change between any mutant FUS as compared to WT FUS (Figure 2.2 A-B), indicating that mutant FUS did not affect the autophagy flux.

2.4.2 FUS knockout reduced the basal level of autophagy

We next examined whether the absence of FUS would affect the autophagy pathway. We used siRNA to knockdown FUS and evaluated the levels of autophagy markers LC3-I and LC3-II. The LC3-II/LC3-I ratio was reduced in the FUS knockdown cells as compared to WT cells (Figure 2.3A). We tested the LC3-I and LC3-II levels in FUS KO N2A cells and obtained a similar result that the LC3-II/LC3-I ratio decreased in the FUS KO cells (Figure 2.3B). Together, these data suggest that FUS might be an important player during autophagy initiation, phagophore elongation, or autophagy flux.

To test whether FUS plays a role in the autophagy initiation, phagophore elongation, or autophagy flux stage, we treated N2A and N2A

FUS KO cells with bafilomycin A1 or rapamycin, followed by Western blots of autophagy markers. Bafilomycin A1 disrupts the late stages of the autophagy flux by inhibiting the autophagosome-lysosome fusion and preventing autolysosome acidification [167]. Consistent with earlier results, the ratio of LC3-II/LC3-I was significantly lower in FUS KO cells as compared to WT cells without Bafilomycin A1 (Figure 2.3C-D). After the Bafilomycin A1 treatment (200 nM, 6 hrs), the LC3-II/LC3-I ratio increased significantly in WT N2A cells (Figure 2.3C, lane 2 vs lane 1) as well as FUS KO cells (Figure 2.3C, lane 4 vs lane 3). However, even in the presence of Bafilomycin A1, the LC3-II/LC3-I ratio was still significantly lower in FUS KO cells compared to WT cells (Figure 2.3C, lane 4 vs lane 2). These results suggest that FUS KO did not affect the late stages of autophagosome-lysosome fusion or autophagy flux, but rather affected the early stages of the autophagy pathway.

Rapamycin is a well-stablished mTOR inhibitor that induces the initiation of autophagy [168]. After the Rapamycin treatment (2 μM, 4 hrs), the LC3-II/LC3-I ratio significantly increased in WT N2A cells as compared to non-treatment (Figure 2.3E-F, lane 2 vs lane 1). In contrast, the LC3-II/LC3-I ratio did not change in FUS KO cells in the presence of rapamycin (Figure 2.3E-F, lane 4 vs lane 3), suggesting that FUS KO cells were not able to respond to the rapamycin-mediated initiation of autophagy.

2.4.3 FUS affected the levels of proteins critical in early phases of autophagy

We next analyzed the expression of proteins involved in the ULK1 initiation complex that is composed of ULK1, Atg13, Atg101 and FIP200. This complex promotes or suppresses the autophagy process depending on the nutrient status of the cell [169]. We tested the expression of Atg13, Atg101, FIP200, total ULK1 and phosphorylated ULK1 in WT and FUS KO N2A cells (Figure 2.4A-B). Atg13, Atg101, total ULK1 expression did not change between WT and FUS KO cells. The phosphorylation on UKL1 Ser757 or Ser555 did not change in FUS KO cells either (Figure 2.4C). Interestingly, FIP200 expression was significantly reduced in FUS KO cells as compared to WT cells (Figure 2.4A-B).

We also evaluated the proteins involved in the phagophore elongation [150]: Atg3, Atg5, Atg12, Atg7 and Atg16L1 (Figure 2.4D-E). Atg3, Atg16L1, and the Atg5-Atg12 complex showed a significant decrease in FUS KO cells as compared to WT cells. However, the protein level of Atg5 alone did not change between FUS KO and WT cells, indicating that only Atg12 protein was affected in the absence of FUS. Similarly, Atg7 did not change in FUS KO cells. Taken together, these results suggest that FUS likely plays an important role in the autophagy initiation and phagophore elongation stages by regulating the expression of FIP200, Atg3, Atg12 and Atg16L1.

2.4.4 FUS regulated the transcription of genes involved in early phases of autophagy

Since FUS regulated the protein levels, we examined whether FUS might regulated the transcription of corresponding genes involved in the initial phases of autophagy using quantitative RT-PCR. For genes involved in the autophagy initiation, the mRNA levels of FIP200 showed a significant decrease (~57%) in FUS KO cells (Figure 2.4F) while Atg101 and Atg7 mRNA levels did not significantly change between the two groups. For genes involved in the phagophore elongation, the mRNA levels of Atg3 (~48%), ATG16L1 (~54%), and Atg12 (~48%) decreased significantly in FUS KO cells (Figure 2.4G). Atg7 was used as a negative control and did not change significantly. These results suggest that FUS likely plays a role in regulating the gene expression of those critical to the autophagy initiation and phagophore elongation.

2.4.5 FUS knock-in restores expression of autophagy related genes.

To further solidify our conclusion, we examined whether re-expression of FUS in the FUS KO cells can restore the expression of the affected autophagy related genes. To this end, we generated a cell line that stably expresses FLAG-tagged WT FUS in N2A-FUS KO cells, hereby named FUS knock-in (KI) cells. The expression level of FUS in the FUS KI cells was comparable in WT N2A cells. FUS KI significantly restored the protein levels of Atg16L1 (100%) and FIP200 (~80%) as compared to FUS KO cells (Figure

2.5A-B). Similarly, FUS KI also increased the mRNA levels of Atg16L1 and FIP200 as compared to the FUS KO cells (Figure 2.5C). The mRNA levels of Atg16L1 and FIP200 were restored to ~60% in WT N2A cells. The results support that FUS plays a role in regulating the transcription of the genes involved in the autophagy initiation and phagophore elongation.

2.5 Discussion

FUS is a DNA/RNA binding protein that has been linked to familial cases of ALS , and its pathology has been reported in FTD patients [114]. Research has found that FUS plays multiple roles in various pathways inside the cell. We hereby report a previously unknown function of FUS, i.e. FUS regulates the initiation of autophagy by mediating the transcription of genes that are critical to autophagosome formation.

We found that the expression of mutant FUS did not change the levels of autophagy markers (Figure 2.2). The role of autophagy in FUS mediated ALS has been debated. One study reported that impairment of the proteasome pathway replicated ALS phenotypes in mice with TDP-43 and FUS proteinopathy, but impairment of the autophagy pathway by motor neuron-specific knock-out of Atg7 did not show ALS phenotype [170]. However, another study showed that torkinib, an autophagy-enhancing compound that functions as an mTOR inhibitor, reduced FUS cytoplasmic aggregates and rescued motor function in P525L iPSC-derived motor neurons [171]. Thus, it

remains possible that both UPS and autophagy pathways contribute to the degradation of toxic protein aggregates in various experimental systems in neurodegenerative diseases [172].

Our results showed that overexpression of mutant FUS did not affect autophagy in N2A cells, which is supported by a study showing that the expression of FUS mutant R521C did not impair autophagy [157]. However, another study reported that overexpressing mutant FUS impairs autophagy by disrupting early stages of Rab-1 dependent autophagy pathway [173]. It is possible that mutant FUS affected the autophagy pathway differently in different experimental systems.

More interestingly, FUS knockdown or knockout cells showed significantly decreased levels of autophagy flux marker (Figure 2.3A-B). Moreover, the FUS knockout cells did not respond to rapamycin treatment as WT cells did (Figure 2.3E-F). Consistently, the level of LC3-II increased in the presence of Bafilomycin A1, but the level in FUS knockout cells was significantly lower than that in WT cells (Figure 2.3C-D). The results suggest that FUS is involved in the LC3-II lipidation, phagophore elongation and autophagosome formation.

Furthermore, we demonstrated that the mRNA and protein levels of FIP200, Atg3, Atg16L1 and Atg12 decreased in FUS KO cells (Figure 2.4), which were restored by expression of FUS (Figure 2.5). ULK1, FIP200 and Atg13 form a complex that is required for ULK1 localization to the isolation membrane and subsequent phagophore formation [174-176]. Atg101 is

associated with the ULK1 complex by interacting with Atg13 [177]. Among the four genes/proteins examined, only FIP200 was affected by FUS KO. FIP200 has been reported to be an essential regulator of autophagy in *Drosophila melanogaster* [178] and mammalian cells [179]. In both studies, when the FIP200 was silenced, the induction of autophagy activity was severely impacted. Thus, we conclude that the regulation of FIP200 expression by FUS is a critical mechanism in autophagy initiation.

Atg (AuTophaGy-related) genes function as a ubiquitin-like conjugation system to facilitate LC3-II lipidation and phagophore elongation [150]. Among five Atg genes examined in this study, the levels of Atg3, Atg16L1 and Atg5-Atg12 complex decreased consistently. The Atg5-Atg12 complex conjugates with Atg16L1 [180] and facilitate the attachment of phosphatidylethanolamine (PE) to the C-terminal glycine of Atg8 (LC3) [149], which promotes phagophore elongation [150, 181, 182]. These studies support our model (Figure 2.6) that FUS regulates the expression of genes critical to LC3 lipidation, phagophore elongation, and autophagosome formation.

It is noted that it is yet to be determined in future studies how FUS regulates the expression of FIP200, Atg3, Atg16L1 and Atg12 genes. FUS was reported to be associated with active chromatin [48], to interact with nuclear receptors [61], transcription factors [60], and RNA polymerase II and the TFIID complex [44], suggesting that FUS is part of the transcription machinery. However, it is unclear how FUS specifically regulate these genes while it does not change other Atg genes. It is conceivable that there are specific elements

in the regulatory region of these genes that specifically interact with FUS. Future studies are needed to determine such FUS-interacting elements for those genes.

Our study discovered a novel function of FUS in protein homeostasis. Specifically, FUS regulates the early stages of autophagy by modulating the transcription of genes that play a role in autophagy initiation (FIP200) and phagophore elongation (Atg12 and Atg16L1). The lack of FUS causes the downregulation of the gene expression and protein levels, leading to decreased phagophore formation, and thus the reduction in basal autophagy flux (Figure 2.6). The function of FUS in autophagy *in vivo* remains to be further examined in future studies. The significance of FUS regulation of autophagy genes in the context of neurodegenerative diseases such as ALS and FTD is also to be better understood.

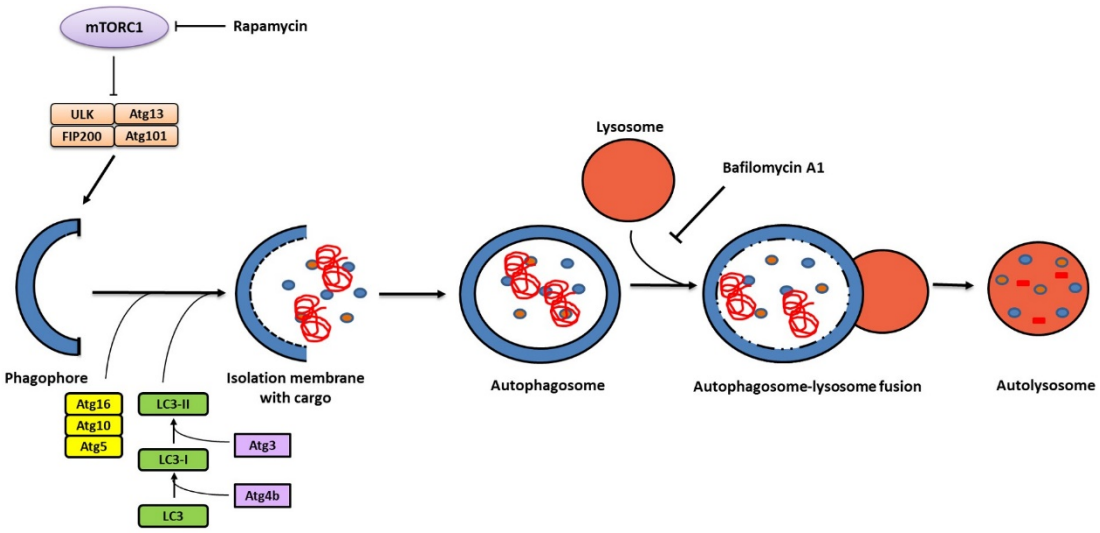

Figure 2.1. The autophagy pathway.

mTORC is a negative regulator of autophagy. Under normal conditions mTORC inhibits autophagy, however under starvation or after rapamycin treatment, mTORC is inhibited and autophagy starts. The ULK initiation complex starts downstream signaling for the generation of the phagophore. Then, the phagophore elongates by the action of Atg12:Atg5:Atg16L and PE-Atg8 conjugation system. The phagophore encloses the cargo that needs to be degraded forming a mature autophagosome that fuses with a lysosome. The lysosome releases acidic proteases that degrade the cargo. Finally, the degraded contents are recycled back to the cell for further metabolism.

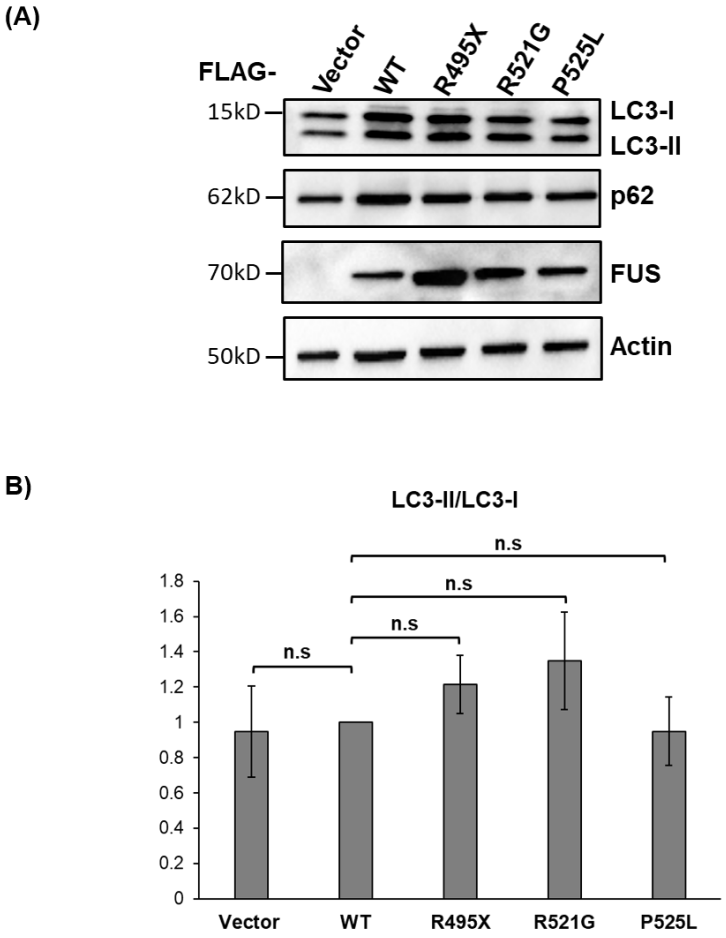

Figure 2.2 Mutant FUS does not affect basal autophagy

(A) FUS knockout N2A cells were transfected with FLAG-tagged vector, WT FUS, FUS-R495X, R521G, P525L. 48h after transfection cells were collected and immunoblotting was performed with the indicated antibodies. (B) Quantification of (A) from three independent experiments ± SD. Student's *t*-test was performed for individual comparisons against FLAG-FUS WT. *, p ≤0.05.

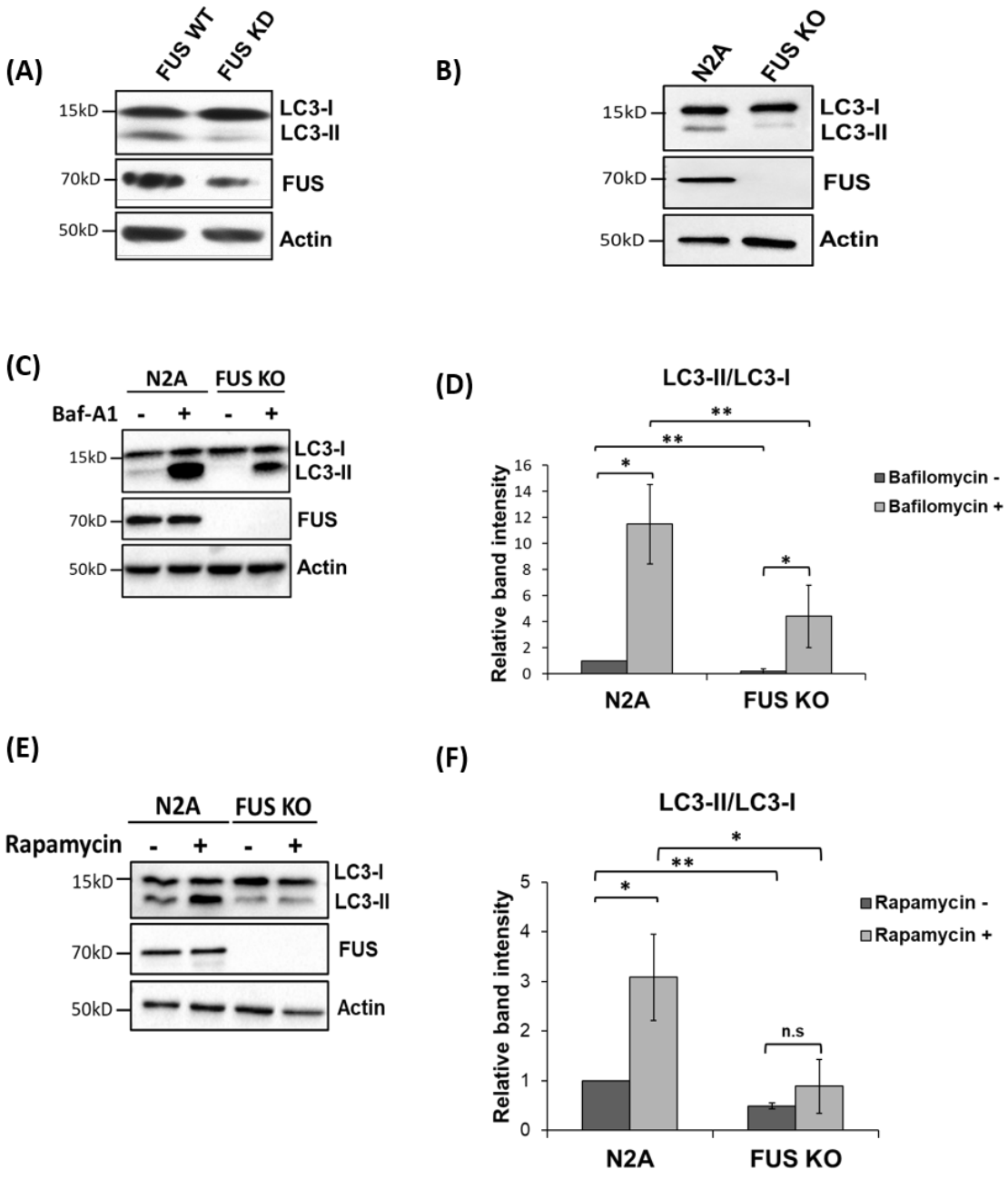

Figure 2.3 Basal autophagy is affected in FUS KO cells

(A) N2A cells were transfected with FUS siRNA using Lipofectamine™ RNAiMAX. 48h after transfection cells were harvested and immunoblotting was performed using the indicated antibodies. (B) N2A and FUS knockout

(KO) N2A cells were cultured and immunoblotting was performed using the indicated antibodies. (C) N2A and KO cells were treated with Bafilomycin A1 (200 nM) for 6h. Cells were harvested, and immunoblotting was performed with the indicated antibodies. (D) Quantification of (C) from three independent experiments ± SD. One Way Anova was performed to determine statistical significance. *p ≤0.05; **p≤0.005. (E) N2A and KO cells were treated with Rapamycin (2μM) for 4h. Cells were harvested, and immunoblotting was performed with the indicated antibodies. (F) Quantification of (E) from three independent experiments ± SD. One Way Anova was performed to determine statistical significance. *p ≤0.05; **p≤0.005.

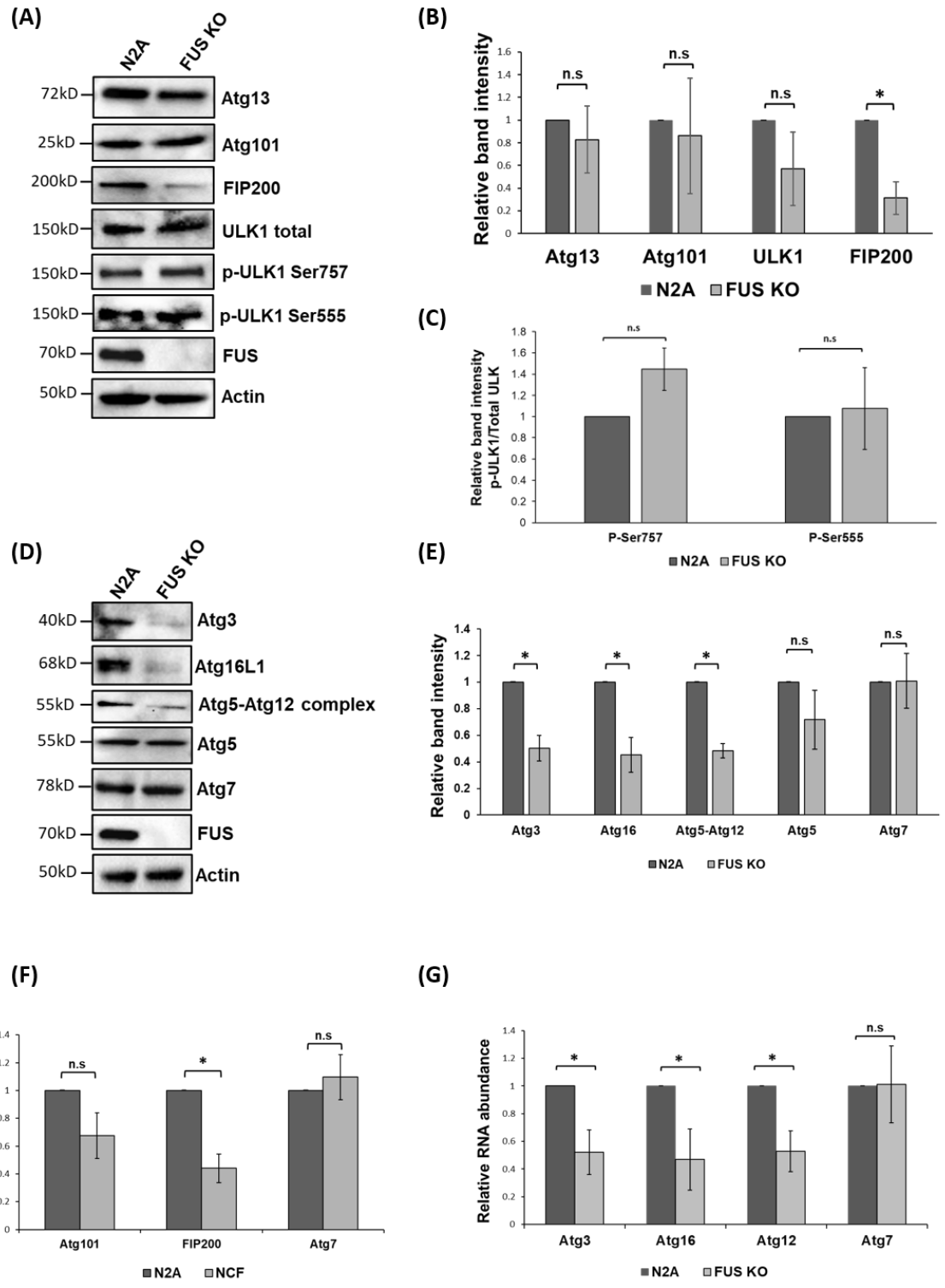

Figure 2.4. FUS regulates transcription of genes involved in autophagy initiation and phagophore elongation

(A) N2A and FUS knockout (KO) N2A cells were cultured and immunoblotting was performed using the indicated antibodies. (B-C) Quantification of (A) from three independent experiments ± SD. Student's *t*-test was performed for individual comparisons against N2A WT cells. *, p ≤0.05. (D) N2A and KO cells were cultured, and immunoblotting was performed using the indicated antibodies. (E) Quantification of (D) from three independent experiments ± SD. Student's *t*-test was performed for individual comparisons against WT N2A WT cells. *, p ≤0.05. (F-G). N2A and KO cells were cultured and harvested for RNA isolation, followed by reverse transcription and quantitative PCR using TaqMan probes for the indicated genes. Actin was used to normalize the protein expression. Averages of three independent experiments are shown, ± SD. Student's *t*-test was performed for individual comparisons against N2A WT cells.

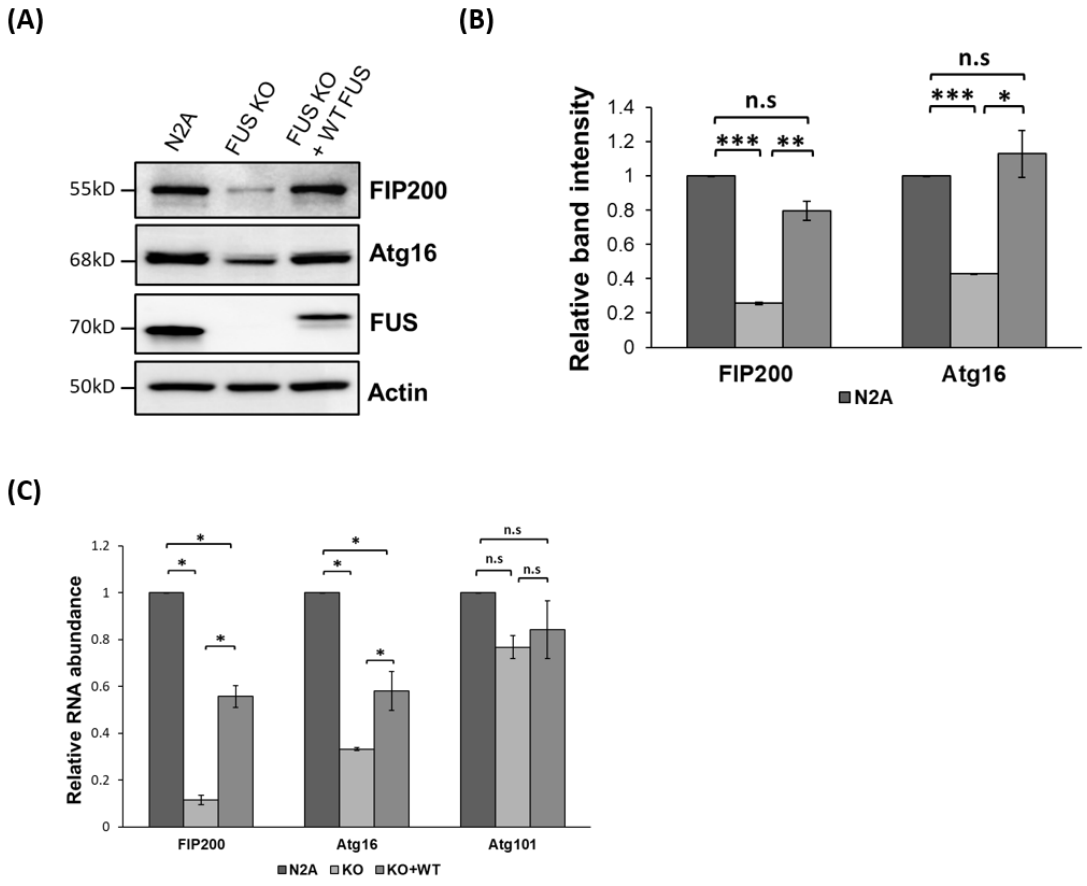

Figure 2.5. FUS knock-in restores expression of autophagy related genes.

(A) N2A, FUS knockout (KO), and FUS knock-in (KO + WT) cells were cultured, and immunoblotting was performed using the indicated antibodies. (B) Quantification of (A) from three independent experiments ± SD. One Way Anova was performed to determine statistical significance. *p ≤0.05; **p≤0.005; n.s: not significant. (C) N2A, KO, KO + WT cells were cultured and harvested for RNA isolation, followed by reverse transcription and quantitative PCR using TaqMan probes for the indicated genes. Actin probe was used to normalize gene expression. Averages of three independent experiments are

shown, ± SD. Student's *t*-test was performed for individual comparisons against N2A WT cells.

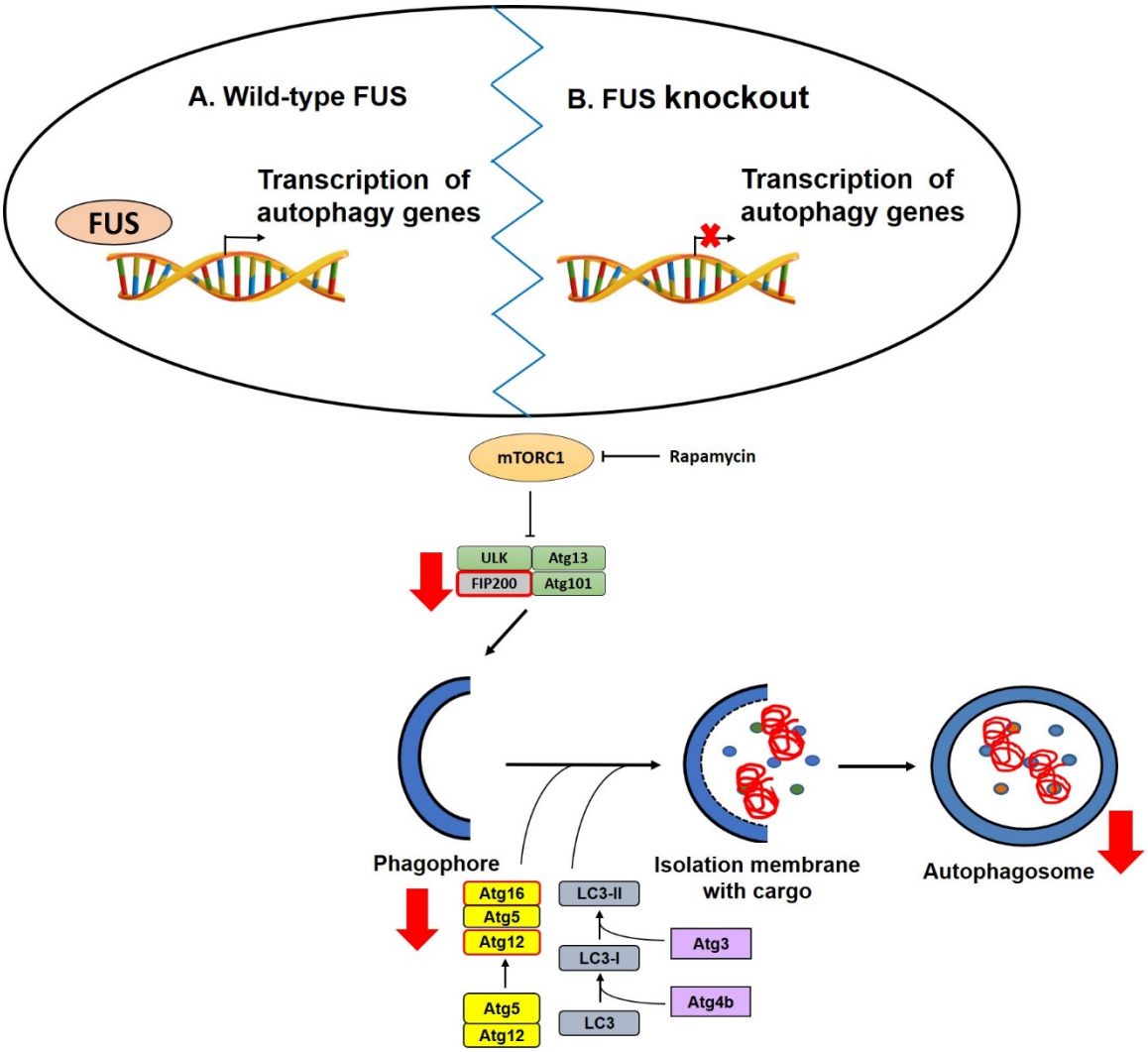

Figure 2.6. Proposed model of the role of FUS in the autophagy pathway

We propose that FUS is involved in transcription regulation of genes involved in autophagy initiation and phagophore elongation. Lack of FUS in the cells causes downregulation at the gene expression and protein levels, causing a decrease in autophagosome formation, and thus the reduction in basal autophagy flux

CHAPTER 3. CONCLUSIONS AND FUTURE DIRECTIONS

Altogether, my work showed two novel characteristics of FUS in the cell. In the first chapter of this dissertation, we showed that FUS can be post-translationally modified by lysine acetylation. Furthermore, we were able to identify the acetylated lysine residues in the RRM domain and C-terminal NLS. We showed that acetylation in K315/K316 in the RRM domain reduced the RNA binding capabilities of FUS. Additionally, we demonstrated that acetylation in the NLS affected the interaction between FUS and Transportin-1, changing FUS subcellular localization and inducing the inclusion formation. Interestingly, we found that ALS patient-derived fibroblasts had increased acetylation at K510, suggesting that the acetylation at this site might be important in the ALS pathology. The acetylation-mimic mutation in the RRM domain and treating cells with the deacetylase inhibitor reduced the number of cells containing cytoplasmic inclusions in cells expressing the ALS mutant FUS P525L. We also found that CBP/p300 acetylated FUS, more specifically K510, and found that HDACs and SIRTs played a role in FUS deacetylation.

Our study shows a novel mechanism by which FUS can be regulated and provides a new avenue for potential therapeutic development in the future. The fact that FUS acetylation on its RRM domain reduces inclusion formation in cells expressing mutant FUS suggests that deacetylase inhibitors could be targeted to this domain in order to prevent aberrant aggregation of FUS in the cytoplasm in ALS patients. In addition, preventing acetylation in the NLS can restore the interaction with Transportin-1 and promote FUS nuclear

localization. Interestingly, we found that ALS patients have higher levels of acetylation at K510. CBP/p300 acetylates this lysine K510 with higher specificity, thus we propose that CBP/p300 inhibitor A-485 might serve as a possible target for future pharmaceutical studies for ALS treatment.

Although this study shows promising findings on FUS regulation, further investigation is needed to characterize the molecular mechanism that controls FUS acetylation and the functional consequences of this PTM in different biological processes where FUS is actively involved. Additional studies are needed to explore the role of FUS acetylation in modulating protein translation and NMD pathway, for instance by testing changes in NMD markers when FUS is acetylated. Since we noticed a change in cytoplasmic aggregation when FUS is acetylated, it would be interesting to examine how acetylation can affect stress granule dynamics, and whether FUS acetylation changes the assembly or disassembly of cytoplasmic aggregates.

The second chapter of this dissertation showed a novel role of FUS in the autophagy pathway. We found that the ALS mutant FUS did not affect autophagy flux. However, FUS KO cells showed decreased levels of LC3-II and were not able to induce autophagy as efficiently as WT N2A cells. We found that proteins involved in autophagy initiation and phagophore elongation were lower in FUS KO cells. Furthermore, we found that the transcription of genes encoding for these proteins were also downregulated. Finally, the protein and mRNA levels of affected genes were rescued when WT FUS was overexpressed in KO cells.

Previous studies had demonstrated that FUS inclusions co-localized with autophagosomes and LC3-II marker, suggesting that cytoplasmic protein aggregates are degraded by the autophagy pathway [157]. However, in this study we found that FUS is a novel player in regulating autophagy. Our data indicate that FUS affects transcription of FIP200 and ATG16L1, however future studies are needed to elucidate the mechanism by which such transcription downregulation occurs. As discussed in Chapter 1, FUS is involved in a variety of nuclear functions, including transcription, splicing, and miRNA processing. All these mechanisms can affect gene expression; thus, it is necessary to perform next generation sequencing in WT and KO FUS cells to differentiate gene expression patterns between these two cell populations. Additionally, it can be useful to determine whether the promoter regions of the autophagy-related genes contain consensus sequences where FUS can bind to and regulate transcription.

The two novel findings described in this dissertation may affect each other although currently there is no evidence. We found that FUS acetylation at K510 disrupts the interaction with TNPO1, preventing FUS to be shuttled into the nucleus and promoting FUS accumulation in cytoplasmic aggregates. A loss-of-function of FUS in the nucleus would disrupt nuclear processes in which FUS plays an important role, including transcription. It is conceivable that a loss-of-function in the nucleus would disrupt the transcription of autophagy genes. Consequently, the autophagy pathway would be impacted,

and the cells will not be able to clear out cytoplasmic aggregates efficiently. This hypothesis can be tested in future studies.

Overall, the two projects described in this dissertation open a new avenue of research that can be further explored and can be translated into therapeutic interventions. Both projects provide novel insights into the role of FUS in neurodegenerative diseases, including ALS and FTD. Further investigation in this field is necessary to find the connection between these two studies, for instance, to explore how the effect of FUS acetylation on protein function and inclusion formation will impact the transcription of genes involved in the autophagy pathway.

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VITA

Education:
2006-2013 B.S. Biology; University of Cauca, Popayan, Colombia.

Positions Held:
2014-2020: Graduate Research Assistant

Awards:

- Molecular Mechanisms of Toxicity, NIEHS Training Grant (2T32ES007266-26A1) 2017-2020
- Lyman T Johnson Fellowship Award (2014 –2017)
- Best Scientific Work in the area of Toxicology. **Maria Alexandra Arenas Guerrero,** María Victoria Ortega Hernandez. XVIII Alexander Hollaender International Course. Asunción, Paraguay. November 10-13, 2013.

Abstracts and Meetings
2018:

- *Emerging role of FUS in the autophagy pathway.*
  Alexandra Arenas, Jozsef Gal, and Haining Zhu.
  Midwest Motoneuron Consortium Biennial Meeting. Hosted by the Indiana University School of Medicine Motoneuron Club.
  Sept 21st-22nd, 2018. Indianapolis, IN, USA. Poster presentation

2017:

- *Characterization of FUS post-translational modifications*.
  Alexandra Arenas, Jozsef Gal, Kelly Barnett and Haining Zhu
  International Symposium on ALS/MND.
  December 8th-10th, 2017. Boston, MA. USA. Poster presentation

2012:

- Frecuencia de Micronucleus en Células del Epitelio Bucal en Personas Ocupacionalmente Expuestas a Solventes Orgánicos**. Arenas Guerrero, Maria Alexandra,** Ortega Hernández María Victoria., Hoyos LS, Marino-Carvajal S. *Memorias del Congreso de Genética.*
  Revista Ciencias de la Salud. Universidad del Rosario Vol. 10(3): 429-664/635. 2012. Printed ISSN: 1692-7273/ Digital ISSN: 2745-4507. Oral presentation

Alexandra Arenas